On the molecular genetic etiology of osteosarcoma

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Scope of the thesis

Theoretically, it was planned to review a start the literature on genes involved in hereditary diseases and on genes involved in the somatic cell processes leading to the development of sporadic osteosarcoma (**Chapter 1**). Experimentally, it was planned to compare gene expression profiles of osteoblasts, the precursor cells of osteosarcoma, osteosarcoma cell lines, representing easily accessible material, and eventually biopsies of osteosarcoma tumours. For this comparison primary osteoblasts should be grown out of bone fragments in culture. Since fibroblasts can grow out of these fragments as well, it was planned to define molecular markers that can be used to fast and efficiently discriminate these cultured osteoblasts and fibroblasts (**Chapter 2**). Osteoblasts are presently characterised by a variety of assays, such as von Kossa staining for the presence of calcium, an alkaline phosphate assay and staining, osteocalcin and collagen assays. These assays are laborious and require considerable numbers of cells. Thus, a rapid and sensitive molecular test would be most welcome. As a first attempt to unravel the genetic aberrations underlying the development of osteosarcoma an identifcation of the gene expression differences between osteoblasts and osteosarcoma cell lines by micro-array analysis was planned (**Chapter 3**). Since possible differences might be either cell culture-specific or tumour-specific, we started collecting primary osteosarcoma tumours to be able to compare osteoblasts, osteosarcoma cell lines and primary osteosarcoma tumours, thus allowing to discriminate between cell culture-specific and tumour-specific expression characteristics (**Chapter 4**). The level of expression of a gene can depend on regulatory mechanisms and/or on its copy number. To assess the influence of the latter, it was planned to also include an array-CGH analysis (**Chapter 4**).

Chapter 1

Introduction

Genes involved in the development of osteosarcoma

I. Incidence, classification and treatment of osteosarcoma

Primary osteosarcoma is a malignant tumour of bone mostly arising in young children and adolescents. The world-wide incidence of osteosarcoma is 1.7 per million in individuals younger than 10 years of age and 8.2 per million in the age group between 10 to 19 years of age (Ragland et al., 2002; Entz-Werle et al., 2003; Sandberg and Bridge, 2003; Nagarajan et al., 2003; Lau et al., 2004). In the Netherlands these incidences are very similar (The Netherlands Cancer Registry 1989-2003). In patients older than 50, osteosarcoma can occur as a consequence of radiation therapy or due to a disease with a high risk of osteosarcoma development, such as Paget disease of bone. Osteosarcoma mostly develops in the second decade of life in the metaphyses of the long bones (femur, tibia, pelvis, ribs and upper arm) that are then rapidly growing in length (Ragland et al., 2002; Kline and Sevier, 2003; Entz-Werle et al., 2003; Sandberg and Bridge, 2003; Lau et al., 2004). About 50% to 70% of cases originate in connections to the knee area (Spina et al., 1998; Marina et al., 2004). The first symptoms of osteosarcoma are pain for several weeks, which can be mild and alternating, a mass or swelling which can be felt at the ends of the long bones. Conventional X-rays will show a bone tumour with irregular borders often with evidence of bone destruction and periosteum reaction. Soft tissue extension is often seen. After the radiological diagnosis of osteosarcoma, histological examination of biopsies is performed to obtain information on malignancy and definitive type of tumour.

Osteosarcoma has a broad histological spectrum. The different types of osteosarcoma have in common a proliferation of malignant mesenchymal stem cells together with the production of osteoid or bone matrix (Benayahu et al., 2001; Marina et al., 2004). Penetration of the tumour into the cortex of the bone leads to bone destruction, which results in a further growth of the tumour into the soft tissue.

Conventional osteosarcoma, the most common type of osteosarcoma, is a primary intramedullary high-grade sarcoma that represents about 75% of all bone tumours (Spina et al., 1998). The World Health Organisation (WHO) recognises three major subtypes of conventional osteosarcoma, namely osteoblastic (50%), fibroblastic (17%), and chondroblastic (33%) (Unni, 1998). Osteoblastic osteosarcoma consists mainly of osteoid or bone as the predominant type of matrix. Chondroblastic osteosarcoma is shaped as lobules of cartilage containing chondrocytes with atypical nuclei in the lacunae. Fibroblastic osteosarcoma is composed of malignant spindle-forming cells with a slight presence of osteoid (Unni, 1998; Spina et al., 1998; Marina

et al., 2004). The remaining 25% non-conventional osteosarcoma are subclassified as telangiectatic osteosarcoma, low-grade osteosarcoma, small-cell osteosarcoma, parosteal osteosarcoma and periosteal osteosarcoma. Telangiectatic osteosarcoma, an aggressive and rare variant, is presented in the second and third decade of life. Characteristic of this type is an osteolytic lesion with no sclerotic changes, preferentially occurring in femur and tibia (Unni, 1998; Spina et al., 1998; Marina et al., 2004). Low-grade osteosarcoma affects slightly older patients (mean age about 30 years). The tumour grows very slowly, so that symptoms persist for a long time and pathologic fractures may occur. Instead of having a fleshy and soft appearance like conventional osteosarcoma, the tumour is firm and fibrous (Unni, 1998; Spina et al., 1998; Marina et al., 2004). Parosteal osteosarcoma, a low-grade osteosarcoma diagnosed in patients in their third decade of life or older, arises from the cortex without invasion in the medullar cavity. In contrast to the conventional osteosarcoma, the parosteal osteosarcoma is clinically indolent and characterised by local recurrence instead of distant metastatic spread after inadequate surgical excision (Unni, 1998; Spina et al., 1998). Small cell osteosarcoma is diagnosed in the long bones at the second and third decade of life by the production of malignant osteoid matrix. Periosteal osteosarcoma is very rare (2%) and its age and gender distributions and clinical features are the same as for conventional osteosarcoma. The difference with conventional osteosarcoma is the site of tumour development, which is the diaphysis instead of the metaphysis of the long bones (Unni, 1998; Spina et al., 1998).

At the time of diagnosis of osteosarcoma almost all patients have already microscopically visible metastases, mostly in the lung. About 80% to 90% of the patients develop metastatic recurrence if they are solely treated with surgical resection and/or radiotherapy (Marina et al., 2004). In general, treatment for patients with osteosarcoma included neoadjuvant chemotherapy with the ultimate goal to reduce the viability of the tumour, to facilitate, in case of an extremity bone sarcoma, limb salvage surgery and to destroy remaining tumour cells. Individual treatment depends on the stage of the disease, the location of the tumour, the presence of metastases, patient's age and general health condition. Patients who achieve 98% of more necrosis of the primary tumour after chemotherapy, have the best prognosis (Kline and Sevier, 2003). Recurrence usually occurs within three years after diagnosis (Kline and Sevier, 2003). The 5-years survival rate of 20% in 1970 changed to the current 55% to more than 70% with neoadjuvant chemotherapy and

surgery (Veth., 1991; Spina et al., 1998; Stock et al., 2000; Gisselsson et al., 2002). Today limb salvage surgery can be achieved in over three quarter of the patients.
Little is known about the molecular etiology of osteosarcoma. Knowing which genes play a role in the development of this tumour might contribute to designing better therapies. The following sections discuss what is known in the literature on genes whose mutations are associated with bone tumourigenesis.

II. Genes whose mutations are associated with a hereditary disease phenotype including osteosarcoma

Osteosarcoma can develop as a consequence of some specific genetic predisposition in patients suffering from retinoblastoma, Li-Fraumeni syndrome, Paget disease of bone, Rothmund-Thomsom syndrome, RAPADILINO syndrome or Werner syndrome (Fuchs and Pritchard, 2002). It cannot be excluded that the genes responsible for these hereditary disorders may also be involved in the development of the much more frequently occurring sporadic osteosarcoma. Therefore, in this section a review is given of what is known about the genes underlying these disorders.

Retinoblastoma

The hereditary form of the eye tumour disease retinoblastoma is one of the hereditary disorders in which osteosarcoma can occur. Retinoblastoma has an incidence of 1/15,000 – 1/20,000 births and is usually diagnosed under the age of 5 (Suckling et al., 1982; Lohmann and Gallie, 2004). All bilateral cases are hereditary. Among unilateral cases about 15% are hereditary. In a minority of retinoblastoma patients, deletions of the long arm of chromosome 13 are present in the germline. These provided a clue to the localisation of the *RB1* gene at band q14 of chromosome 13 (Lee et al., 1987). Osteosarcoma is the most common secondary neoplasm of patients with the hereditary form of retinoblastoma. It occurs in particular, though not exclusively, in the region exposed during radiotherapeutic treatment of retinoblastoma, namely the head and neck region (Radig et al., 1998; Chauveinc et al., 2001; Sandberg and Bridge, 2003; Lohmann and Gallie, 2004). The incidence of osteosarcoma as a second primary tumour in patients with retinoblastoma is approximately 12% (Hansen, 1991; Thomas et al., 2001).

Li-Fraumeni syndrome

Li-Fraumeni syndrome is a rare cancer predisposition syndrome caused by germline mutations in the *TP53* (Tumour Protein 53) gene located at 17p13.1.The tumours associated with Li-Fraumeni syndrome, such as soft-tissue and bone sarcomas, brain tumours, adrenocortical tumours, breast cancer and leukaemia, occur over a wide age range, including childhood (Siddiqui et al., 2005). The incidence of osteosarcoma as a second cancer in patients with Li-Fraumeni syndrome is approximately 12% (Hisada et al., 1998).

Paget disease of bone

Paget disease of bone is an autosomal dominant hereditary condition affecting 3% - 5% of individuals older than 55 (Haslam et al., 1998; Hansen et al., 1999; Good et al., 2001; Reddy, 2004). The frequency in males is higher than in females (1.8:1) (Nellissery et al., 1998; Hansen et al., 1999; Good et al., 2001). The disease is characterised by an imbalance between formation and degradation of bone, leading to a disorganised bone structure with an increased risk of pathological fractures. The bones mostly affected are pelvis, femur, tibia, lumbar spine, sacrum and skull (Nellissery et al., 1998; Hansen et al., 1999). Genetic factors are also involved in the pathogenesis of related syndromes such as juvenile Paget disease of bone, early-onset Paget disease of bone, familial expansile osteolysis and expansile skeletal hyperphosphatasia. Osteosarcoma develops in 1% of patients with Paget disease of bone, i.e. at a rate which is a thousand-fold higher than in the general population (Hansen et al., 1999; Fuchs and Pritchard, 2002; Reddy, 2004; Takata et al., 2004). Osteosarcoma tumours associated with Paget disease of bone are characterised by the presence of a large number of osteoclastic giant cells and atypical osteoblasts (Roodman and Windle, 2005).

Paget disease of bone segregates often as an autosomal dominant trait manifesting genetic heterogeneity and incomplete penetrance (Haslam et al., 1998; Hocking et al., 2000; Laurin et al., 2001; Good et al., 2001; Reddy, 2004). The two loci first claimed as being involved in Paget disease of bone were PDB1 at 6p21.3 (Fotino et al., 1977) and PDB2 at 18q21-22 (Haslam et al., 1998). They may be restricted to only a few families since involvement of both loci could not be confirmed in several studies involving a large number of families with Paget disease of bone (Hocking et al., 2000; McNairn et al., 2001; Laurin et al., 2001; Good et al., 2001). That led to a

genome-wide scan of in total 24 French Canadian families and to the mapping of two novel loci for Paget disease of bone, at 5q31 and 5q35-qter (Laurin et al., 2001). Searching for genes at 5q35-qter, the PDB3 locus (~300 kb), resulted in two genes of interest. One is *SQSTM1*, which mediates intracellular signalling through the IL-1/TNF pathway toward NF-κB. The other gene is *MAPK9,* a potential component of the RANK-signalling pathway. No sequence variations were found in *MAPK9*. In 11 families tested, the same *SQSTM1* mutation was detected, namely a P392L mutation in exon 8 (Laurin et al., 2002). This mutation was also described in families of different origin (Hocking et al., 2002; Good et al., 2004). Five other *SQSTM1* mutations were identified in familial Paget disease of bone individuals, namely two resulting in a premature stopcodon, one in a transversion, one in an inversion at exon 7 and one in an insertion at exon 8. All these mutations cluster near the C-terminal of the protein product p62 and are predicted to disrupt the ubiquitin binding properties of *SQSTM1* (Hocking et al., 2002; Johnson-Pais et al., 2003b; Good et al., 2004).
As the remaining 13 French Canadian families investigated by Laurin et al. (2001) did not harbour any mutation in the *SQSTM1* coding sequence, it is likely that also other disease genes are associated with Paget disease of bone. In two of the 13 families the locus PDB4 at 5q31 seemed to be involved (Laurin et al., 2001). Moreover, there is evidence for three other loci associated with the disorder: PDB5 at 2q36 (Hocking et al., 2001), PDB6 at 10p13 (Hocking et al., 2001) and PDB7 at 18q23 (Good et al., 2002).

Rothmund–Thomson syndrome

Rothmund-Thomson syndrome is an autosomal recessive disorder with a heterogeneous clinical profile characterised by poikiloderma, growth deficiency, premature ageing and an increased predisposition to osteosarcoma (Kitao et al., 1999a; Wang et al., 2001). Rothmund-Thomsom syndrome has some clinical similarities with Werner syndrome and with Bloom syndrome, which have been shown to be due to mutations in *RECQ3* helicase and *RECQ2* helicase, respectively. Based on these findings, *RECQ* genes were considered as candidate genes for patients diagnosed with Rothmund-Thomson syndrome. Kitao et al. (1999) were the first to report that patients from families with Rothmund-Thomson syndrome had mutations in the RecQprotein-like 4 gene (*RECQL4),* located at 8q24.3. Involvement of *RECQL4* has been reported for a proportion of patients diagnosed with Rothmund-Thomson syndrome (Kitao et al., 1999b; Lindor et al., 2000; Wang et al., 2003).

Osteosarcoma occurs only in those patients of Rothmund-Thomson syndrome who have truncating mutations in *RECQL4*. For these patients the risk of developing osteosarcoma is 5% (Wang et al., 2001; Wang et al., 2003).

RAPADILINO syndrome

RAPADILINO syndrome is another rare disease, clinically showing some overlap with Rothmund-Thomson syndrome, but with a general absence of poikiloderma. The acronym RAPADALINO stands for the features: RAdial hypoplasia/aplasia, PAtellar hypoplasia/aplasia and cleft or highly arched PAlate, DIarrhea and DIslocated joints, LIttle size (> 2SD below the mean of height) and LImb formation, NOse slender and NOrmal Intelligence. The RAPADILINO syndrome is an autosomal recessive disease with a frequency of 1:75,000 in Finland, the country with the highest prevalence of this disease. Patients diagnosed with RAPADILINO have also truncated mutations in the *RECQL4* gene. Phenotypic correlation with *RECQL* gene defects shows that the osteosarcoma incidence in RAPADILINO syndrome is over 4 times lower than in Rothmund-Thomson syndrome (Siitonen et al., 2003; Kellermayer et al., 2005).

Werner syndrome

Werner syndrome or adult progeria is an autosomal recessive disease characterised by premature ageing, skin atrophy, hair loss, greying hair and atherosclerosis. The incidence of Werner syndrome is low: about 23 new cases a year worldwide, most of these in Japan (Satoh et al., 1999). The first symptoms present in the second decade of life, during which patients lack the pubertal growth spurt. The syndrome is caused by mutations in the *WRN* (*RECQL2)* gene, located at the chromosomal region 8p11-p12 (Puzianowska-Kuznicka and Kuznicki, 2005). In addition to the aforementioned characteristics, patients with Werner syndrome have a high risk of developing tumours, such as osteosarcoma, soft-tissue sarcoma, thyroid carcinoma, melanoma and menigioma (Shimamoto et al., 2004). Whereas in the general population carcinomas are more common than sarcomas (ratio of 10:1), patients with Werner syndrome have carcinomas of epithelial origin and sarcomas of mesenchymal origin occurring at similar frequencies (Chen and Oshima, 2002; Hauben et al., 2003; Puzianowska-Kuznicka and Kuznicki, 2005). Osteosarcoma, soft-tissue sarcoma, melanoma and thyroid cancer constitute 57% of all cancers in Werner syndrome (Goto et al., 1996). The primary sites of osteosarcoma in Werner syndrome patients

are in the lower extremities (ankle/foot), whereas in the general population they usually occur in the upper extremities (Ishikawa et al., 2000; Chen and Oshima, 2002).

III. Somatic-genetic aberrations in sporadic osteosarcoma: Studies on the chromosome level

Because of the rarity of the hereditary diseases of which osteosarcoma development can be part of the clinical phenotype, the large majority of osteosarcoma tumours occur as sporadic osteosarcoma. They show multiple genomic aberrations that have a somatic origin, i.e. they do not occur in the germline. These aberrations have been investigated by a variety of methods on both the chromosomal level and the gene level. There can be a relationship between these two levels in the sense that tumour development can be promoted by loss of chromosomal regions containing genes with a tumour suppressor function or by gain of chromosomal regions containing genes functioning as oncogenes, though it should be realised that tumour development can also be either stimulated or inhibited by many genes with other functions, e.g. in DNA repair, immune responses, cell cycle regulation, apoptosis, etc. An analysis of chromosome alterations in sporadic osteosarcoma can, therefore, provide a clue to the location of genes involved in the development of osteosarcoma.

Conventional cytogenetic analysis of primary tumours

The first cytogenetic analysis of primary osteosarcoma tumours were single case reports describing a triploid-tetraploid range (Mandahl et al., 1986;Castedo et al., 1988) karyotype with numerous abnormalities. Karyotypes of primary osteosarcoma tumours are very complex with great variation in chromosome numbers and with many rearranged chromosomes (Mertens et al., 1993; Hoogerwerf et al., 1994). What most tumours have in common is that at least one of the normal chromosomes 13 is missing (Mandahl et al., 1986; Castedo et al., 1988; Biegel et al., 1989; Hoogerwerf et al., 1994). Frequently, there is also loss of one chromosome 17 and a high incidence of structural rearrangements of 17p11~13 (Toguchida et al., 1992; Miller et al., 1996b; Bridge et al., 1997).

The most common numerical abnormalities in primary osteosarcoma tumours are duplications of chromosome 1, loss of chromosomes 9, 10, 13 and 17 and partial or complete loss of chromosome 6. The most common rearrangements involve chromosomal bands or regions 1p11~p13, 1q10~q12, 1q21~q22, 11p14~p15, 12p12, 17p11~p13, 19q13 and 22q13 (Biegel et al., 1989; Mertens et al., 1993; Bridge et al., 1997; Zielenska et al., 2001; Sandberg and Bridge, 2003).
Osteosarcoma cell lines, derived from primary osteosarcoma, also exhibit a high degree of numerical and structural rearrangements (Bayani et al., 2003; Lim et al., 2005). Ozaki et al (2003) investigated four cell lines by multicolour karyotyping and found many more frequently involved - i.e. occurring in two or more of the four cell lines - structural rearrangements, including 1p31, 1q22, 1q31, 1q41, 4q32, 6q22~6q24, 8q24, 9q22, 9q33, 10p12~p13, 11p13~p14**,** 11q23~q24, 12p12, 13q12~q13, 15q13~q14, 17p11~p12, 17q21~q22, 21q11 and 22q12~22q13. Based on the described chromosomal rearrangements, primary osteosarcoma tumours and osteosarcoma cell lines have the regions 1q22, 11p14, 12p12, 17p11~p12 and 22q13 in common.

Molecular analysis of allelic imbalances

Losses of heterozygosity or allelic imbalances most commonly observed in osteosarcoma involve the long arm of chromosome 13 and the short arm of chromosome 17 (Hansen, 1991; Scheffer et al., 1991; Feugeas et al., 1996; Wolf et al., 1999; Entz-Werle et al., 2003), fully in agreement with the findings reported for conventional cytogenetic analysis. Applying loss of heterozygosity analysis to primary high-grade osteosarcoma from 54 patients, Entz-Werle et al. (2003) found additional loss of the regions 5q21, 7q31 and 9p21. Yamaguchi et al. (1992) investigated DNA from 37 patients with primary conventional osteosarcoma and reported allelic losses at seven chromosome arms: 3q, 6p, 10q, 13q, 15q, 17p and 18q. The highest frequency of allelic loss in this study was observed at chromosome 3q. Allelic losses at 10q were investigated in more detail by Mendoza et al. (2005). They showed loss of heterozygosity for marker D10S1723 at 10q26 in 60% of the investigated osteosarcoma (Yamaguchi et al., 1992; Mendoza et al., 2005). Loss of heterozygosity has also been reported for the short arm of chromosome 18, between the polymorphic markers D18S60 and D18S42 that colocalise with the *PDB2* locus which is involved in Paget disease of bone (Nellissery et al., 1998; Patino-Garcia et al., 2003; Johnson-Pais et al., 2003a).

Frequent loss of heterozygosity of all or part of a chromosome arm in osteosarcoma may point to tumour suppressor gene inactivation and can thus indicate the location of a tumour suppressor gene. The most frequent losses of heterozygosity in osteosarcoma involve 3q, 13q, 17p and 18q.

Comparative genomic hybridisation

For comparative genomic hybridisation (CGH), total genomic DNA is isolated from test and reference samples, labelled with two fluorochromes, and hybridised to a representation of the genome, which allows the binding of sequences at different locations to be distinguished. In the first CGH studies, metaphase chromosomes were used for the representation of the genome. Nowadays, mostly DNA micro-arrays are used. Regions of gain (duplication, amplification) or loss (deletion) of DNA sequences, are seen as changes in the ratio of the intensities of the two fluorochromes (Kallioniemi et al., 1992; du Manoir et al., 1993; Pinkel and Albertson, 2005).

Although comparative genomic hybridisation of normal metaphase spreads has a limited resolution (~10-15 megabases) because of its dependence on the morphology of metaphase chromosomes, application of this method using labelled DNA from primary osteosarcoma tumours has revealed that the regions most frequently involved in chromosomal losses are 2q34-qter, 3p, 3qcen-q22, 5q, 6q16-qter, 8p12-pter, 9p13-pter, 10p12, 10q23, 11p12-pter, 13qcen-q21, 14q, 15qcen-q21, 16p, 17p and 18q. The most common chromosomal gains have been detected at 1p21-p31, 1p35-p36, 1q21-q31, 2p, 2q31-32, 3q25-qter, 4q12-q13, 4q27-q32, 5p13-p15.2, 5q, 7q31-q32, 8q21.3-q22, 8qcen-q13, 9q21-22, 11q14, 14q24-qter, 16p, 17p, 19(p), 20q, 21q, Xpcen-p21 and Xq25-qter. High-level amplifications occurred at 1p22-p31, 1q21-q24, 3q26, 6p12-p21.3, 8q23-q24, 12q12-q13, 12q14-q15, 17p11-p12 and Xp11.2-p21. (Forus et al., 1995; Tarkkanen et al., 1995; Tarkkanen et al., 1998; Tarkkanen et al., 1999; Zielenska et al., 2001; Ozaki et al., 2002).

Osteosarcoma cell lines rather than primary tumours are commonly used for investigations because they can easily be cultured. Only a few osteosarcoma cell lines have so far been analysed by CGH. Losses appeared most frequently at 3p12-p14, 7q33-qter, 18q12, 18q21–q22 and 19p. Gains were most frequently observed at 1p21-q24, 1p22-1p31, 1q25-q31, 5p, 6p21.2-p22, 6q22, 7p21, 7p22, 7q31, 8q23-q24, 9q21, 12p12, 14q21 and 14q22-qter. High-level gains were detected at 1p21-p22, 5p, 6q12-q15, 8q23-q24 (Ozaki et al., 2003). Based on the described

chromosomal rearrangements, primary osteosarcoma tumours and osteosarcoma cell lines have as losses in common the regions 3p12-p14, 18q12, 18q21-q22 and as gains the regions 1p22-p31, 1p21-q24, 1q25-q31, 5p, 7q31, 9q24 and 14q24-qter. For the two chromosomal regions 1p22 and 8q23-q24, high-level amplification was detected in both primary osteosarcoma tumours and osteosarcoma cell lines.

Array based comparative genomic hybridisation

Instead of a spread of metaphase chromosomes an array of mapped human genomic DNA fragments (~100-200 kb) can be used for hybridisations (Pinkel et al., 1998). This allows a high-resolution and high-throughput quantitative measurement of DNA copy number changes throughout the genome. Different micro-arrays can be used, such as arrays produced from large-insert genomic clones (PACs and BACs), cDNAs, selected PCR products and oligonucleotides (Pinkel and Albertson, 2005).

Primary osteosarcoma tumours

In the first genome-wide array comparative genomic hybridisation on osteosarcoma, 48 tumours were hybridised on an array consisting of 967 human BACs, spaced at approx. 3 Mb across the whole genome (Man et al., 2004). The most frequent deletions were observed at the chromosomal regions 2q31.1, 3p14.1, 4p16.2, 6q12, 6q21, 7q35, 10p15.1, 10q22-q23, 10q25-q26, 11q25, 13q12.2, 13q14.3, 13q22.1, 17p13.3 and 17q12. The chromosomal regions that represented gains were mapped to 1p36, 4p16, 6p12-p21, 8q21, 8q23-24, 12q14.3, 16p13.3, 17p11-p12, 19p13.3 and 21q22.3. Among this limited number of tumour samples, 7 recurrent homozygous deletions were found that mapped to 1q25.1, 3p14.1, 4p15.1, 6q12, 6q13 and 13q12.22 (Man et al., 2004).

Squire et al. (2003) analysed 7 tumours using a cDNA micro-array of 18,980 human clones. Except for the chromosomal losses as also observed by conventional comparative genomic hybridisation, additional losses were detected at 1q, 6q, 13q11-q21 and 20q. Those genes at 6q and 13q are in agreement with the findings by Man et al. (2004) for these chromosomal regions. Amplifications were observed for the chromosomal regions 17p12-p13 and 8q23-q24. So far, two other studies (Zielenska et al., 2004; Atiye et al., 2005) have reported high-resolution analysis of osteosarcoma on cDNA arrays of about 18,980 genes and 13,000 genes respectively. Amplified regions were detected at 1p34-p35, 1q21, 6p12-p13, 8q24, 12q11-q15, 17p11-p13, 19q13 and 21q22 by Atiye et al. (2005). Zielenska et al.

(2005) reported as amplified regions 1q21-q22, 12q13-q15 and 17p11.2. With the exception of genes at 1q21, amplified genes at all the other regions are in agreement with the findings by Man et al. (2004) with respect to gains of chromosomal regions.

Osteosarcoma cell lines

BAC arrays have been used by Lim et al. (2004) for an analysis of chromosomal aberrations of the osteosarcoma cell line MG-63, which is commonly used in experimental model studies. With a small array of only 1400 clones high-level amplifications were observed for the chromosomal regions 6p21, 6p22~pter and 8q23~q24 (Lim et al., 2004). With an array of 32,433 clones similar and additional amplifications were observed for the chromosomal regions 6p21, 8q24 and 9p21-p22 (Lim et al., 2005).

Previously described chromosomal aberrations identified by conventional cytogenetic analysis and comparative genomic hybridisation on metaphase spreads have been confirmed and extended by array comparative genomic hybridisation. The high-resolution of the produced micro-arrays allows precise localisation of DNA copy number alterations, thus pinpointing possible specific target genes that play a role in the development of osteosarcoma.

IV. Somatic-genetic alterations in sporadic osteosarcoma: Studies on the gene level

From the studies on the chromosome level, multiple genes appear to be involved in the development of sporadic osteosarcoma. They can largely be divided in tumour suppressor genes, whose physical and functional loss will stimulate tumour development and oncogenes, whose activation and amplification will do the same. Amplifications of oncogenes represent a late feature, associated with tumour progression rather than initiation (Barrios et al., 1993; Ladanyi et al., 1993b). Table 1 gives an overview of genes that have been studied in connection with osteosarcoma development.

A. Tumour suppressor genes

RB1

Retinoblastoma 1 (*RB1*) was the first tumour suppressor gene to be cloned and functions as the major regulator of the G1 to S phase progression in the cell cycle (Friend et al., 1986; Ragland et al., 2002). The sporadic form of retinoblastoma depends on first a somatic mutation of one copy of *RB1* in a cell of the developing retina and subsequently a mutation of the second *RB1* copy in a cell derived from cells carrying the first mutation. The cell-cycle regulatory pathway centered on the retinoblastoma protein, pRb, is inactivated in most human cancers (Weinberg, 1995), including retinoblastoma, osteosarcoma, small cell lung carcinoma and bladder carcinoma. During cell division *RB1* binds to and thereby suppresses the function of E2F transcription factors, the ability to activate transcription of proteins important for DNA synthesis. The binding of *RB1* to E2F is controlled by phosphorylation that is mediated by D-type cyclin-dependent kinases, in particular CDK4/cyclin-D complex (Thomas et al., 2001; Ragland et al., 2002; Knudson, 2002). Loss of a putative cell-cycle specific function of pRB disturbs the process of cell-cycle control (Weinberg, 1995; Miller et al., 1996b; Fuchs and Pritchard, 2002).

In 50% to 70% of sporadic osteosarcoma cases, heterozygous loss of the chromosomal region 13q14, comprising the RB1 locus, is observed (Yamaguchi et al., 1992; Feugeas et al., 1996). The fact that 13q belongs to the chromosomal arms most frequently involved in losses of heterozygosity, as mentioned earlier, indeed indicates the presence there of a tumour suppressor gene, c.q. *RB1*. High frequency of loss of heterozygosity might be an early predictive feature for patients with osteosarcoma, indicating a potential unfavourable outcome (Fuchs and Pritchard, 2002; Patino-Garcia et al., 2003). Structural rearrangements of *RB1* are seen in about 30% of sporadic osteosarcoma (Wadayama et al., 1994; Miller et al., 1996b). Point mutations of the *RB1* gene have been found in less than 10% of sporadic osteosarcoma (Wadayama et al., 1994). *RB1* alterations occur more frequently in high-grade osteosarcoma than in low-grade osteosarcoma (Wunder et al., 1991).

Whereas in view of the specific association of osteosarcoma with the hereditary form of retinoblastoma *RB1* might have some still not elucidated tissue-specific function, the fact that the gene is involved in most human cancers seems to contradict a very specific role of *RB1* in the development of sporadic osteosarcoma.

Table 1: Genes claimed as being involved in the development of sporadic osteosarcoma.

Gene Symbol [1)]	*Gene coding for*	*Location*	*Previous Symbol*	*Aliases*
CDK4	Cyclin-Dependent Kinase 4	12q13		PSK-J3
CDKN2A	Cyclin-Dependent Kinase inhibitor 2A	9p21	*CDKN2, MLM*	CDK4I, $p16^{INK4}$, INK4a, MTS1, CMM2, ARF, p19, p14, INK4, $p16^{INK4A}$
CDKN2B	Cyclin-Dependent Kinase inhibitor 2B	9p21		$P15^{INK4B}$, MTS2, INK4B
COPS3	COP9 constitutive photomorphogenic homolog subunit 3 (Arabidopsis)	17p11.2		SGN3
DCC	Deleted in Colorectal Cancer	18q21.1		
GLI1	Glioma-associated oncogen homolog (zinc finger potein)	12q13 – q14	*GLI*	
MAPK7	Mitogen-Activated Protein Kinase 7	17p11.2	*PRKM7*	BMK1, ERK5
MDM2	Mdm2, transformed 3T3 double minute 2, p53 binding protein (mouse)	12q13-q14		HDM2
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	8q24		c-MYC
PMP22	Peripheral Myelin Protein 22	17p12-p11.2		HNPP, GAS-3, SP110
PRIM1	Primase, polypeptide 1, 49kDa	12q13 or 1q44		
RB1	Retinoblastoma 1 (including osteosarcoma)	13q14.2	*OSRC*	
TSPAN31	Tetraspanin 31	12q13-q15	*SAS*	
TOP3A	Topoisomerase (DNA) III alpha	17p12-p11.2		TOP3
TP53	Tumour Protein 53 (Li-Fraumeni syndrome)	17p13.1		P53

[1)] (http://www.gene.ucl.ac.uk/nomenclature/)

TP53

TP53 (Tumour Protein 53) is also one of the most frequently mutated genes in cancer in general. *TP53* is located at 17p13, encodes a 53-kDa nuclear phosphoprotein (Mulligan et al., 1990) and acts as a “guardian of DNA”, activated after cellular stress, such as DNA damage, aberrant proliferative signals, heat shock or hypoxia. The wild-type TP53 protein regulates genes involved in DNA repair, cell cycle arrest, and programmed cell death (Pakos et al., 2004). In human cancers, such as osteosarcoma, colorectal cancer, breast cancer and bladder cancer, cells with mutant *TP53* accumulate genomic alterations, such as genome-wide allelic imbalances, karyotypic abnormalities and chromosomal amplifications. This suggests that the loss of p53 function contributes to tumourigenesis by destabilisation of the genome (Overholtzer et al., 2003).

In osteosarcoma, *TP53* is identified as abnormal in 40% to 60% of cases, mainly high-grade osteosarcoma (Overholtzer et al., 2003 ; van Dartel and Hulsebos,

2004a). On average, osteosarcoma with *TP53* mutations has a mean instability score of 19.1 chromosomal changes per tumour in contrast to 11.1 chromosomal aberrations for almost any other cancer (Overholtzer et al., 2003). The mechanisms of inactivation of the *TP53* gene in osteosarcoma can be divided into three groups: allelic loss in 75% to 80% of the tumours, gene rearrangements in 10% to 20%, and point mutations in 20% to 30%. Point mutations are predominantly missense mutations whose products can form heterodimers with and thereby inactivate normal p53 molecules (Ragland et al., 2002; Sandberg and Bridge, 2003). Again, the high level frequency of allelic losses involving 17p indicates the presence of a tumour suppressor gene on that arm, c.q. TP53.
Given the fact that *TP53* is also a gene generally involved in human cancers, a very specific role in the development of osteosarcoma is not likely.

DCC

One of the identified loss of heterozygosity regions in osteosarcoma is the long arm of chromosome 18. At 18q21.1 a supposed tumour suppressor gene, *DCC* (Deleted in Colorectal Cancer), resides. It was originally identified in allelic loss studies of colorectal cancer (Fearon et al., 1990). *DCC* encodes a transmembrane neural cell adhesion molecule processes with a relatively low expression at the cell surface and is involved in cellular differentiation and developmental processes. So far, only a single study has appeared describing the reduced or lost expression of *DCC* in high-grade osteosarcoma and osteosarcoma cell lines based on *DCC* RNA and protein expression analyses (Horstmann et al., 1997). With a 1.65 kb *DCC* cDNA probe no rearrangements or deletions in osteosarcoma were found. This probe did, however, not fully constitute the 4.34 kb open reading frame of *DCC* and thus neither rearrangements outside this region nor subtle alterations within it would be detected. Present evidence does not justify considering *DCC* as a tumour suppressor gene (Horstmann et al., 1997).

CDKN2A and CDKN2B

Cell-cycle regulation is an equilibrium between cyclin-cyclin-dependent kinase complexes and a family of cyclin-dependent kinase inhibitors that bind cyclin-cyclin-dependent kinase complexes and block their kinase activity. Cyclin-dependent kinase inhibitors block the G1 to S transition and thus the initiation of DNA synthesis and proliferation. Two cyclin-dependent kinase inhibitors, *CDKN2A* and *CDKN2B* are classified as tumour suppressor genes. They have both been localised at

chromosome 9p21, a region identified by rearrangements or deletions in various kinds of human cancers, including osteosarcoma (Miller et al., 1996a; Patino-Garcia and Sierrasesumaga, 1997).

CDKN2A expresses two mRNA transcripts. The alpha variant encodes the $p16^{INK4}$ protein, which acts as a cyclin D/CDK4 inhibitor preventing phosphorylation of pRB and blocking G1 to S transition and cell growth (Patino-Garcia and Sierrasesumaga, 1997; Nielsen et al., 1998; Benassi et al., 1999; Benassi et al., 2001). The beta transcript encodes $p14^{ARF}$, which can interact with the oncogene *MDM2* and consequently causing a block of p53 degradation and an enhanced p53-dependent activation, resulting in an increased G1 phase arrest or apoptosis (Benassi et al., 2001).

CDKN2B encodes the protein $p15^{INK4B}$, which can bind and inhibit *CDK4* and *CDK6* and therefore acts as a cyclin-dependent kinase inhibitor similar to $p16^{INK4}$. Inactivation of *CDKN2A* and *CDKN2B* by homozygous deletions or mutations may affect the balance of the cell-cycle regulatory pathway involving $p16^{IKN4}$, cyclin D, CDK4 and pRB, leading to abnormal cell growth (Miller et al., 1996a).

CDKN2A and *CDKN2B* are less frequently mutated in sporadic osteosarcoma than *RB1* and *TP53,* with percentages of 5% to 10%. This low rate of alterations of *CDKN2A* and *CDKN2B* is consistent with the infrequent loss of heterozygosity affecting chromosome 9p21 in sporadic osteosarcoma (Yamaguchi et al., 1992). In osteosarcoma cell lines, however, *CDKN2A* and *CDKN2B* show mutations in up to 60%. This may suggest that they probably have a more specific role in tumour cell growth in culture (Miller et al., 1996a).

B. Oncogenes

MYC

MYC is the myelocytomatosis viral oncogene homolog (avian) normally involved in the regulation of cell proliferation, DNA replication and the regulation of transcription of specific genes (Ladanyi et al., 1993b). Dysregulation of *MYC* can occur by different mechanisms, such as rearrangement, amplification and point mutation (Ladanyi et al., 1993b). As a consequence proliferation increases, cell differentiation is blocked and may induce apoptosis (Pompetti et al., 2003).

Overexpression of both *MYC* mRNA and protein was observed in 9 of 21 osteosarcoma specimens tested (Gamberi et al., 1998), in particular in those that developed metastases and in the metastases themselves, suggesting that *c-MYC*

overexpression is involved in the progression of osteosarcoma (Gamberi et al., 1998). In a number of studies *MYC* amplification has been investigated by Southern blot hybridisation. Three to fourfold *MYC* amplifications were found in proportions that varied between 2 out of 27 (7%) and 2 out of 4 (50%) of tested osteosarcoma specimens (Bogenmann et al., 1987; Ikeda et al., 1989; Ladanyi et al., 1993b; Pompetti et al., 1996).

GLI1

The glioma-associated oncogene homolog *GLI1* has been mapped to the long arm of chromosome 12 (12q13.3-q14.1). It was originally identified in human glioblastoma, in which it was more than 50-fold amplified (Kinzler et al., 1987; Roberts et al., 1989). The DNA sequence of *GLI1* predicts a gene product related to the *Krüppel* family of zinc finger proteins, implying that *GLI1* may bind nucleic acid sequences involved in gene regulation. *GLI1* is not a member of any known oncogene family, but its possible interfering with gene regulation might lead to tumourigenesis (Roberts et al., 1989). The possibility that *GLI1* could be involved more in general in the malignant progression of childhood solid tumours led to its analysis in primary osteosarcoma, where a 15-fold amplification of *GLI1* was detected (Roberts et al., 1989; Khatib et al., 1993).

TSPAN31/SAS

TSPAN31, a member of the tetraspanin family, also known as *SAS,* is a large superfamily of membrane proteins that are involved in various cell-cell and matrix-cell interactions, including cell adhesion, migration, signal transduction, activation, proliferation and differentiation (Wunder et al., 1999; Huang et al., 2005). The gene *TSPAN31/SAS* is located at 12q13-q14. Amplification of *TSPAN31/SAS* occurs more frequently in parosteal osteosarcoma (low-grade) than in high-grade intramedullary osteosarcoma and other kind of tumours, such as malignant fibrous histiocytoma, a rhabdomyosarcoma cell line, lipoma and liposarcoma (Noble-Topham et al., 1996; Ragazzini et al., 1999). When amplification of *TSPAN31/SAS* occurs in low-grade osteosarcoma, it is tightly linked with an increased expression of *CDK4* and shows co-amplification with this gene (Tarkkanen et al., 1998; Wunder et al., 1999; Gamberi et al., 2000).

MDM2

Mouse Double Minute 2 homolog (*MDM2*), located at 12q13-q14, encodes a protein that can bind p53 and Rb and inactivate p53. When that occurs, $p21^{WAF1}$, which normally binds to cyclin D/CDK4 complexes, will be lower expressed and thereby block the DNA replication and cell cycle progression. This implies that alterations of *MDM2* can have an impact on the p53 and pRb growth control mechanisms (Ragazzini et al., 1999; Wunder et al., 1999). Overexpression of the *p53*-binding *MDM2* gene, located at 12q13-q14, has been reported in 14% to 27% of the recurrent and metastatic lesions of osteosarcoma (Ladanyi et al., 1993a; Forus et al., 1995; Tarkkanen et al., 1995; Tsuchiya et al., 2000) and in 4% to 7% of primary osteosarcoma (Nakayama et al., 1995; Miller et al., 1996b; Lonardo et al., 1997; Park et al., 2004). Thus, amplification of *MDM2* may signal or promote tumour progression in some cases of osteosarcoma (Ladanyi et al., 1993a; Yokoyama et al., 1998). Amplification of *MDM2* goes very often together with amplification of *SAS*, because both genes lie close together in this region of chromosome 12 (Wunder et al., 1999).

CDK4

Cyclin Dependent Kinase 4 (*CDK4*), located at 12q13, is involved in phosphorylation and inactivation of pRb, which leads to cell cycle progression (Ragazzini et al., 1999; Wunder et al., 1999). Overexpression of *CDK4* may result in uncontrolled cell proliferation (Wunder et al., 1999). Expression of *CDK4* was identified first in an osteosarcoma cell line with a 25-fold amplification of the gene (Khatib et al., 1993). Co-amplification with *TSPAN31/SAS* has been found in 12% to 100% of primary parosteal osteosarcoma (Tarkkanen et al., 1998; Wunder et al., 1999; Gamberi et al., 2000). In high-grade osteosarcoma *CDK4* amplification occurs rarely or not at all (Wei et al., 1999; Wunder et al., 1999).

PRIM1

DNA Primase I *(PRIM1*), a DNA-dependent RNA polymerase that can initiate or prime DNA synthesis, is involved in the G1 to S transition of the cell cycle. It maps to 12q13 or 1q44 (Cloutier et al., 1997; Yotov et al., 1999). *PRIM1* appeared to be amplified in 9 of the 22 osteosarcoma tumours tested (Yotov et al., 1999). So far, no more data have been published on *PRIM1* amplification in osteosarcoma.

In osteosarcoma, amplification of the q13-q15 region on chromosome 12 occurs frequently, consequently leading to higher copy numbers of the proto-oncogenes

GLI, SAS, MDM2, CDK4 and *PRIM* that lie in this region. This regional amplification is found more frequently in parosteal (low-grade) osteosarcoma than in the more common intramedullary (high-grade) type of osteosarcoma (Ragazzini et al., 1999; Wunder et al., 1999).

PMP22

In 8 of 9 osteosarcoma specimens tested, van Dartel et al. (2004) found *PMP22* at 17p12-p11.2 higher expressed than in normal osteoblasts. In four of the cases this higher expression was associated with amplification of the gene. In the other four, the higher expression apparently was induced by other mechanisms. In a mixed sample collection of primary tumours and metastases amplification levels greater than 4 were detected in 9 of 19 cases (van Dartel et al., 2002). In normal tissue, the PMP22 protein product is involved in cell growth regulation (van Dartel and Hulsebos, 2004b). It is called the peripheral Myelin Protein 22 (*PMP22*) since it is highly expressed in peripheral Schwann cells.

MAPK7

The mitogen-activated protein kinase 7 gene, *MAPK7,* located at 17p11.2 is involved in signal transduction pathways and triggered by growth factors that induce cell proliferation. It appeared amplified in 10 of 19 tested osteosarcoma tumours and metastases (van Dartel et al., 2002). A test of expression levels by macro-array showed that for *MAPK7* expression in osteosarcoma was not significantly different from osteoblasts, which makes it unlikely that *MAPK7* should be considered a candidate oncogene (van Dartel et al., 2004).

TOP3A

The topisomerase (DNA) III alpha gene *TOP3A* at 17p12-p11.2 is amplified in 13 of 19 high-grade osteosarcoma cases analysed (van Dartel et al., 2002). The expression level of *TOP3A* was also tested by macro-array. It appeared that the expression levels of the tested osteosarcoma samples and osteoblasts were not different from these of the other genes present on the macro-array (van Dartel et al., 2004), in contradiction with the high percentage of amplified osteosarcoma tumours as described by van Dartel et al. (2002). This makes *TOP3A* less likely as a true amplification target in the 17p region (van Dartel et al., 2004).

COPS3

The gene *COPS3*, encoding a component of the proteasome pathway, namely cop9 signalosome-specific phosphorylation, targets *TP53* to *MDM2*-mediated ubiquitination and subsequent degradation by the 26S-proteasome (Henriksen et al., 2003). Amplification of *COPS3* has been found in 20% to 44% of primary osteosarcoma (Henriksen et al., 2003; van Dartel et al., 2004) and in 4% of metastastic cases investigated (Henriksen et al., 2003). Overexpression of *COPS3* causes an increased degradation of the TP53 protein resulting in an increased genomic instability (van Dartel and Hulsebos, 2004a). Tumours with amplification of *COPS3* did not show amplification of *MDM2* and/or mutations in *TP53* in the same tumour sample (Henriksen et al., 2003). This is consistent with array-CGH data, where amplifications of 12q13-q15 and 17p11-p12 were never observed in the same tumour sample (Forus et al., 1995; Stock et al., 2000).

Comparative genomic hybridisation studies have shown that in 13% - 29% of high-grade osteosarcoma the region 17p11.2-p13 was amplified (Forus et al., 1995; Tarkkanen et al., 1995; Tarkkanen et al., 1999; Man et al., 2004). Amplification of this region has also been identified in tumour types such as high-grade gliomas, leiomyosarcomas, malignant fibrous histiocytomas, chondrosarcomas and oral squamous cell carcinomas. The diversity of tumours in which the region 17p11.2 ~p12 is amplified suggests presence in this region of an oncogene or oncogenes more widely involved in tumourigenesis (van Dartel et al., 2002). This 17p region harbours the genes *PMP22*, *TOP3A, COPS3, MAPK7* and *ERBB2.* Because of their high degree of amplification, *PMP22* and *COPS3* seem to be the most likely oncogene candidates.

Conclusion

In primary osteosarcoma many chromosomal abnormalities occur, as shown by conventional cytogenetic analysis, losses of heterozygosity studies and comparative genomic hybridisation. Primary osteosarcoma tumours have frequent losses at 2q31.1, 3p12-p14, 4p16.2, 6q12, 6q21, 7q35, 10p15.1, 10q22-q23, 10q25-q26, 11q25, 13q12.2, 13q14.3, 13q22.1, 17p13.3, 17q21, 18q21-q22 and 20 and gains at 1p22, 1p36, 6p12-p21, 8q21, 8q23-q24, 12q14, 16p13, 17p11-p12, 19p1, 21q2. For two chromosomal regions, 1p22 and 8q23-q24, high-level amplification has been detected in both primary osteosarcoma tumours and osteosarcoma cell lines. *RB1* at

13q and *TP53* at 17p are involved in most human cancers and may therefore, not be attributed a specific role in osteosarcoma development. For the genes *DCC, CDKN2A* and *CDKN2B* real evidence is lacking to consider them as tumour suppressor genes involved in the development of osteosarcoma. Amplifcation of the q13-q15 region on chromosome 12 occurs frequently in osteosarcoma. The proto-oncogenes *GLI, TSPAN31/SAS, MDM2, CDK4* and *PRIM1* map to this region. The region 17p11.2-p13 is amplified in 13% - 29% of high-grade osteosarcoma. Although this region is amplified in other human cancers as well and several genes are mapped to this region, the genes *PMP22* and *COPS3* are the most likely oncogene candidates. So far, many studies have been published describing genetic changes presumably contributing to the development of osteosarcoma. In an attempt to further clarify this process we have performed gene expression profiling and array-CGH on osteosarcoma cell lines as well as primary osteosarcoma, as described in the following part of this thesis.

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Chapter 2

A fast and efficient gene expression test to discriminate between osteoblasts and fibroblasts in culture

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Abstract

Under specific culturing conditions cells growing out from bone fragments are considered to be osteoblasts. Collagenase pre-treatment is generally applied to the bone fragments to get rid of fibroblast admixture. Since morphologically the cultured osteoblasts cannot be discriminated from fibroblasts, we developed an efficient test to discriminate between both cell types. We compared gene expression profiles of 10 osteoblast and 11 skin fibroblast cultures using a 21K oligonucleotide micro-array. This comparison resulted in 42 genes showing a significantly different expression between the two types of cultured cells. These differentially expressed genes were specifically related to membrane proteins/processes such as cell communication, cell adhesion and receptor activity. Expression differences for six of these genes defined by micro-array analysis were confirmed by real-time RT-PCR. *VCAM1, KIAA1644, FGFR2* and *COL27A1* had a higher expression in osteoblasts, whereas *IMP-3* and *MME* were higher expressed in fibroblasts. Since for all six genes differences between all pairs of individual cultures were fully consistent, expression analysis of these genes provides a fast and efficient test to discriminate between osteoblasts and fibroblasts.

Introduction

Specific conditions for *in vitro* culture of cells out of trabecular bone fragments have been determined such that preferentially cells considered as osteoblasts are obtained. Pre-treatment of the bone fragments with collagenase is used to remove fibroblasts. The effectiveness of this collagenase treatment is hard to directly assess, since both cell types, osteoblasts and fibroblasts, are morphologically similar. The cultured cells are, therefore, in general characterised by a variety of assays, such as the von Kossa staining on the presence of calcium, an alkaline phosphatase assay and staining, osteocalcin and collagen assays (Coelho et al., 2000; Coelho and Fernandes, 2000; Martinez et al., 2001). These assays require a considerable number of cells. It is, therefore, common practice to stimulate osteoblastic cell cultures with cytokines such as *BMP-2* and *TGF-β*, or to obtain cells from multiple passages (Han et al., 2002; Vaes et al., 2002). An alternative to discriminate osteoblasts from fibroblasts may be offered by gene expression profiling with micro-arrays. This approach has here been applied to define molecular markers that can be used to distinguish osteoblasts from fibroblasts. We selected six genes, *VCAM1, KIAA1644, FGFR2, COL27A1, IMP3* and *MME* that showed a consistent difference between osteoblast and fibroblast cultures with micro-array based gene expression profiling and real-time RT-PCR analysis.

Materials and methods

Patient material

Eight female and two male individuals undergoing total knee replacement surgery with average ages of 71 and 67 years, respectively, were included in this study as donors of trabecular bone fragments. For fibroblasts, human fibroblast cell cultures of passages 4 to 10 were grown from skin biopsies of 11 different individuals. All samples were collated fully anonymous and used in accordance with the ethical regulations of the hospital.

Isolation of osteoblasts and fibroblasts

Trabecular bone fragments were treated as described by Scheven et al. (1991). The specimens were transported under sterile conditions from the operating centre to the laboratory, chopped into pieces of 0.2 cm –1.0 cm in diameter and washed three times with sterile phosphate-buffered saline. Bone fragments were treated with collagenase (1 mg/ml) (GIBCO – Invitrogen Inc, Carlsbad, Ca, USA) at 37°C during 2.5 hours and again washed three times with sterile phosphate-buffered saline. Subsequently, the bone fragments were cultured in Dulbecco‘s modified Eagle medium (DMEM) with l-glutamax supplemented with 100 IU/ml penicillin and 1000 µg/ml streptomycin (all from GIBCO - Invitrogen) and 10% foetal bovine serum (Biowhittaker – Cambrex, Verviers, Belgium), in small culture flasks (25 cm^2) at 37°C in a humidified atmosphere with 5% CO_2.

Skin fibroblasts were seeded into 25 cm^2 culture flasks and were cultured in Ham`s nutrient mixture F12 with l-glutamine (GIBCO) supplemented with 200 mM l-glutamine, 100 IU/ml penicillin, 1000 µg/ml streptomycin and 250 µg/ml fungizone amphotericine B (GIBCO - Invitrogen) and 10% foetal bovine serum. To account for differences in culture medium, four of the fibroblast isolations were in parallel grown in osteoblast-specific medium. After having reached 70% - 80% of confluence, osteoblasts and fibroblasts were harvested by trypsin (0.05%)/EDTA (0.02%) (GIBCO - Invitrogen) treatment, pelleted, washed with sterile phosphate-buffered saline and stored at –80°C until RNA isolation.

RNA isolation

Total RNA was isolated with the RNeasy mini kit (Qiagen, Valencia, CA, USA) and subsequently treated with DNase (Qiagen) as described by the manufacturer. Total RNA yield and purity were calculated by measuring absorbance’s at 260 nm and 280 nm spectrophotometrically on a Nanodrop® ND-1000 UV-VIS Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA integrity and size distribution was determined by 1% formaldehyde agarose gel electrophoresis.

mRNA amplification and labelling

Linear amplification of mRNA was performed essentially according to a protocol of the Dutch Cancer Institute (www.nki.l.nl/nkidep/pa/microarray/protocols.htm). Briefly, amplification started with first strand cDNA synthesis from 2 µg of total RNA, using Superscript II RT-polymerase (GIBCO - Invitrogen Inc, Carlsbad, Ca, USA) and a specific oligo(dT) primer containing a 17bp T7 polymerase recognition site (5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG$[T]_{24}$-3') (Eurogentec, Seraing, Belgium). After second strand synthesis, double-stranded cDNA was purified with the Qiaquick PCR purification kit (Qiagen) and the yield was determined spectrophotometrically. *In vitro* transcription was performed with the T7 Megascript kit (Ambion, Huntingdon-Cambridgeshire, UK) as described by the manufacturer. However, instead of UTP, a 1:1 mixture of aminoallyl-UTP (Ambion) and UTP was used with a final concentration of 7.5 mM for all NTPs as described by 't Hoen et al. 2003. Amplified RNA (aRNA) was purified with the RNA clean up protocol (Qiagen). Five µg of aRNA was labelled by coupling monoreactive Cyanine-3 or Cyanine-5 fluorophores (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) to the aminoallyl-modified nucleotides. Labelled aRNA was separated from unincorporated Cyanine 3 or Cyanine 5 molecules over Microspin G50 columns (Amersham Biosciences) as described by the manufacturer.

Experimental design

For the identification of genes differentially expressed between osteoblasts and fibroblasts, a common reference design was applied. The common reference sample consisted of an equal mixture of total RNA of 5 fibroblast and 5 osteoblast samples. Amplified RNA from each of the 11 fibroblast and 10 osteoblast samples were labelled with Cyanine-3 and amplified RNA from the reference sample with Cyanine-5, resulting in a total of 21 micro-array hybridisations (Table 1).
To determine the effect of differences in culture medium, effects of osteoblast-specific medium and fibroblast-specific medium on expression profiles were directly compared for four fibroblast samples in a non-reference design including dye swap hybridisations, accounting for 8 micro-array hybridisation reactions in total (Table 1).

Table 1: Overview of hybridisation designs for both the osteoblast-fibroblast comparison and culture media experiments.

Experiment	*Cyanine-3*	*Cyanine-5*	*Number of arrays*
osteoblast-fibroblast comparison			
	osteoblast cultures 1 – 10	reference pool	10
	fibroblast cultures 1 – 11	reference pool	11
culture media comparison			
	fibroblast medium	osteoblast medium	4
	osteoblast medium	fibroblast medium	4

Micro-array slides & hybridisation

In-house manufactured human oligonucleotide arrays were used containing the Qiagen 21,329 human gene specific 70-mer oligonucleotide set version 2.1 extended with 4,000 negative and positive control features. The oligonucleotides were printed in a concentration of 10 pM on Ultra-GAPS amino-silane coated slides (Corning, New York, USA) using BioRobotics 10K quill pins with a MicroGrid spotter (Isogen). Blocking, prehybridisation and hybridisation were performed as described by Hegde et al (2000) with some modifications. In short, slides were blocked with ethanolamine at 52°C during 1 h. Prehybridisation was done with prewarmed prehybridisation buffer containing 0.5% Bovine Serum Albumin (Sigma-Alldrich, St Louis, MO, USA) at 52°C during 45 min. Subsequently, the slides were washed 6x in preheated water (52°C), dried by centrifugation at 800 rpm during 3 min and immediately used for hybridisation. The hybridisation sample consisting of a fluorescently labelled probe mixture and 30 µg poly-A (Sigma Alldrich) was mixed with an equal volume of preheated (52°C) 2x hybridisation buffer. The hybridisation sample was heated at 95°C for 3 minutes before it was applied to the preheated slides. Hybridisation was performed in hybridisation chambers (Telechem International Inc, Sunnyvale, CA, USA) in a waterbath at 52°C in the dark for approximately 48 h. Subsequently, slides were washed with five wash solutions under agitation: 1xSSC/0.2% SDS at 52ºC; 0.1xSSC/0.2% SDS at 52ºC; 0.1xSSC at 52º; 0.1xSSC at RT and 0.01xSSC at RT. Each wash step lasted 5 min. Finally slides were dried by centrifugation at 800 rpm during 3 min and scanned with an Affymetrix GMS428^{TM} array scanner.

Micro-array data analysis

Fluorescent signal intensity data for each spot and for each fluorophore were extracted from the scanned images of each micro-array slide using ImaGene version 5.6 (BioDiscovery, El Segundo, California, USA). Signal intensity data were log transformed and for each spot the Cyanine-5/Cyanine-3 signal intensity ratio was determined and subjected to print-tip normalisation using the Limma package from the Bioconductor project in the statistical programming environment R (Smyth et al., 2005). Normalised data was further analysed using BRB ArrayTools v3.1 developed by Dr. Richard Simon and Amy Peng Lam (http://linus.nci.nih.gov/~brb/download.html). Basically, data was vigorously filtered to exclude control spots, empty spots, spots with high inter-spot intensity variability and spots designated as bad by eye. Genes that had more than 25% missing data across all observations after filtering were also excluded from the analysis. Genes significantly differentially expressed between the osteoblast and fibroblast cell cultures were identified by an F-test using a randomised variance model. Moreover, a multivariate permutation test was applied to account for the proportion of the number of false discoveries in the set of significantly differentially expressed genes (Reiner et al., 2003). Besides this class comparison, several multivariate class prediction algorithms, such as the Compound Covariate Predictor, Diagonal Linear Discriminant Analysis, Nearest Neighbour Classification and Support Vector Machine Predictor (discussed in Simon et al., 2003a and 2003b) were applied to the micro-array data to identify a set of genes predicting whether a sample belonged to either the osteoblast or the fibroblast group (Zhao et al., 2003). To determine the accuracy of the outcome of each prediction algorithm, the leave-one-out cross-validation misclassification rate and the significance of this misclassification rate were estimated by random permutation of the class phenotypes (osteoblasts versus fibroblasts) and reanalysing from the start for 2000 permutations for each of the algorithms. To identify genes that were influenced by the culture media, resulting log-ratio data were analysed by the one-sample t-test with the null hypothesis that the mean of the log-ratio distribution is equal to zero. In addition, an overrepresentation analysis of gene ontology (GO) categories (http://www.geneontology.org) was performed by EASE (http://david.niaid.nih.gov/david/ease.html). Using the most recent data release of the GO annotations, representation of each GO category in the filtered list of genes and selected gene set was compared. Based on the EASE score for significance and the

Bonferroni correction for multiple testing, significant GO categories were identified (Hosack et al., 2003).

Real-Time RT-PCR

For real-time PCR, cDNA was generated using Ready-To-Go™ You-Primed First-Strand Beads (Amersham Biosciences) and an oligo-dT primer (Eurogentec) as described by the manufacturers. The endogenous control β2-microglobulin was selected by analysing an osteoblast sample, a fibroblast sample and two osteosarcoma samples for different housekeeping genes on a Taqman Human Endogenous Control Plate (Applied Biosystems, Foster City, California, USA) as described by the manufacturer. For the genes to be analysed, primer-probe sets were obtained as an assay-on-demand system (Applied Biosystems).
Total RNA from 10 individual osteoblast cultures and 11 individual fibroblast cultures was analysed in triplicate in a final volume of 15 μl in a 384-well plate with the ABI Prism 7900 Sequence Detection System (Applied Biosystems). Real-time data were analysed with the Sequence Detection System software (SDS2.1.1, Applied Biosystems). The threshold value was calculated as ten standard deviations from the baseline with a maximum threshold cycle number (Ct) value for the baseline of 15. By setting the threshold and baseline, the Ct value for each replication of each sample was extracted from the respective amplification plot. For each gene the difference in expression between the osteoblast and fibroblast samples was determined using the ΔΔ median Ct method. The corresponding fold change in expression was calculated by the formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen., 2001).
Real-time RT-PCR data was statistically analysed using ANOVA, including the β_2-microglobulin gene as a covariate in the model.

Results

Identification of differentially expressed genes between osteoblasts and fibroblasts

We used 21K oligonucleotide micro-arrays to compare gene expression profiles of 10 osteoblast and 11 fibroblast samples in a common reference design (Table 1). Statistical analysis showed that among the remaining 2643 genes after filtering 120 genes were significantly ($P < 0.001$) differentially expressed between osteoblasts and fibroblasts with a false discovery rate (FDR) of 6%. Three of the four class prediction methods used showed that this set of 120 genes predicted the correct class for each sample with high accuracy ($P < 0.0005$) based on leave-one-out cross validation. For only one of the osteoblast samples the predictive value of the Support Vector Machines algorithm was insufficient.

From the 46 most significant genes ($P < 0.0001$, FDR < 1%) potential marker genes were selected (Table 2). Thirty of these 46 genes had a higher expression in osteoblasts than in fibroblasts, with fold differences ranging from 1.5 to 12.5. The remaining 16 genes showed higher expression levels in fibroblasts with fold differences ranging from 1.5 to 9.

To exclude that the observed differences between osteoblasts and fibroblasts were due to the differences in culture medium, four of the fibroblast cultures were cultured in both osteoblast-specific medium and fibroblast-specific medium. Statistical analysis showed that 80 genes were differentially expressed between fibroblasts from the same isolations grown on either cell culture medium, but at a relatively low level of significance ($P <0.01$), due to the small size of the experiment. Only two genes, *TXNIP* (NM_006472) and *TARSH* (NM_015429) were highly significantly ($P < 0.000026$) influenced by the cell culture medium. These genes had a five fold and a two fold higher expression in the fibroblast-specific F12 medium, respectively. The set of 80 genes, as identified to be differentially expressed, between both culture media showed, only a marginal overlap of four genes with the 120 genes differentially expressed between osteoblasts and fibroblasts. These four genes, including *TXNIP*, belonged to the 46 most significantly differing genes between the osteoblast and fibroblast samples (Table 2).

From the remaining 42 genes, we selected four genes that were more abundantly expressed in osteoblasts, namely *VCAM1, KIAA1644, FGFR2,* and *COL27A1* (Table

2A) and two genes that were more abundantly expressed in fibroblasts, *IMP3* and *MME* (Table 2B). Selection criteria were a fold difference of at least 3 and as far as applicable a known role in osteoblast-related biology. Micro-array results for these genes were validated by applying real-time RT-PCR to the same genes.

Table 2A: Significantly (*P* <0.001) higher expressed genes in cultured osteoblasts than in cultured fibroblasts.

GenBank Accession	*Gene coding for*	*Gene Symbol*	*Fold Difference*
NM_001078 [c]	Vascular cell adhesion molecule 1	*VCAM1*	12.5
AB051431 [b,c]	KIAA1644 protein	*KIAA1644*	7.6
NM_000596	Insulin-like growth factor binding protein 1	*IGFBP1*	5.9
NM_015865 [b,c]	Solute carrier family 14 (urea transporter), member 1	*SLC14A1*	5.9
NM_005252 [a]	V-Fos FBJ murine osteosarcoma viral oncogene homolog	*FOS*	5.6
NM_000493 [d]	Collagen, type X, alpha 1	*COL10A1*	5.1
AK025719	FLJ 22066 fis, clone HEP10611	*Na*	4.7
NM_023028	Fibroblast growth factor receptor 2	*FGFR2*	4.4
AY027862	Upregulated in colorectal cancer gene 1	*UCC1*	3.9
NM_019111 [b,c]	Major histocompatibility complex, class II, DR alpha,	*HLA-DRA*	3.9
NM_020474	UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase 1	*GALNT1*	3.8
NM_005261	GTP binding protein overexpressed in skeletal muscle (GEM), transcript variant 1	*GEM*	3.8
AL359052 [a]	cDNA clone EUROIMAGE 1968422	*Na*	3.8
BC008442 [b,c]	Transmembrane 4 L six family member 1	*MGC14656*	3.8
AK021858	cDNA FLJ11796 fis, clone HEMBA1006158, highly similar to Homo sapiens transcription factor forkhead-like 7 (FKHL7) gene	*FOXC1*	3.7
AF278532 [d]	Na	*Na*	3.5
AF216077	Collagen, type XXVII, alpha 1	*COL27A1*	3.4
NM_003937	Kynureninase (L-kynurenine hydrolase)	*KYNU*	3.2
NM_004791 [b,c,d]	Integrin, beta-like 1 (with EGF-like repeat domains)	*ITGBL1*	3.1
NM_002133	Heme oxygenase (decycling) 1	*HMOX1*	3.0
NM_000095 [d]	Cartilage oligomeric matrix protein	*COMP*	2.9
AL389943 [a]	cDNA clone EUROIMAGE 2005779	*Na*	2.8
NM_000956 [b,c]	Prostaglandin E receptor 2 (subtype EP2),	*PTGER2*	2.5
NM_001912	Cathepsin L	*CTSL*	2.5
NM_002829	Protein tyrosine phosphatase, non-receptor type 3	*PTPN3*	2.4
M68874	Phosphatidylcholine 2-acylhydrolase	*PLA2G4A*	2.4
AL157424	Synaptojanin 2	*SYNJ2*	2.2
AF220656	Pleckstrin homology-like domain, family A, member 1	*PHLDA1*	2.1
AK055484	cDNA FLJ30922 fis, clone FEBRA2006485	*SXBP5*	2.1
AF247704	NK3 transcription factor related, locus 1 (Drosophila)	*NKX3-1*	2.1
NM_005245 [b,c]	FAT tumour suppressor homolog 1 (Drosophila)	*FAT*	2.0
NM_005544	Insulin Receptor Substrate 1	*IRS1*	2.0
NM_002160	Na	*Na*	2.0
NM_006644	Heat shock 105kDA/110kDA protein 1	*HSPH1*	1.9
NM_000428 [d]	Latent transforming growth factor beta binding protein 2	*LTBP2*	1.8
NM_005857	Zinc metalloproteinase (STE24 homolog, yeast)	*ZMPSTE24*	1.5

Genes marked by [a] are genes influenced by culture media differences. Genes belonging to GO cellular component category integral to plasma membrane are marked by [b], plasma membrane by [c] and extracellular matrix by [d].

Table 2B: Significantly (*P* <0.001) lower expressed genes in cultured osteoblasts than in cultured fibroblasts.

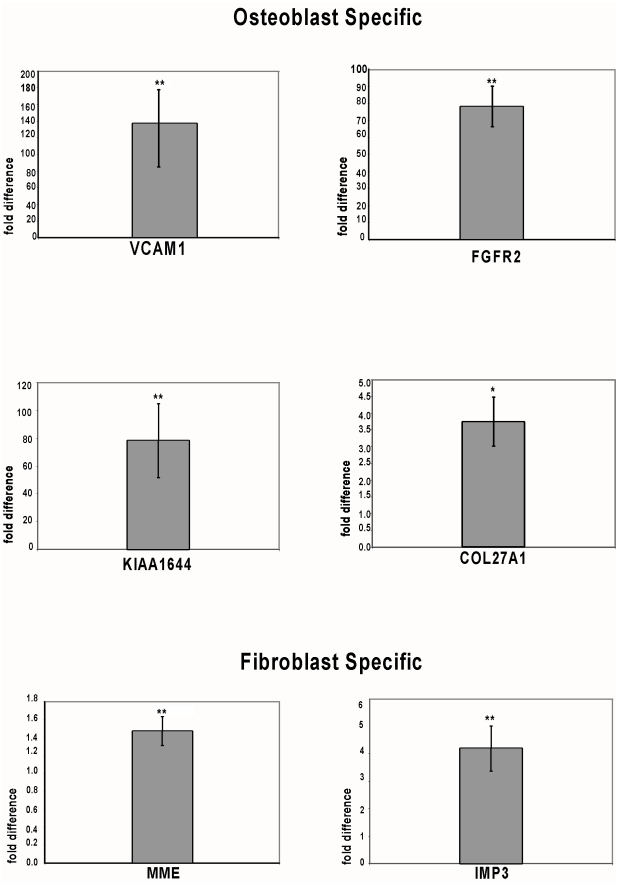
GenBank Accession	*Gene coding for*	*Gene Symbol*	*Fold Difference*
NM_006472 [a]	Thioredoxin interacting protein	*TXNIP*	9.1
NM_013381 [b,c]	thyrotropin-releasing hormone degrading enzyme	*TRHDE*	6.2
NM_007289 [b,c]	Membrane-Metallo-Endopeptidase	*MME*	5.6
NM_006547	IGF-II mRNA-binding protein 3	*IMP-3*	4.3
NM_000121 [c]	Erythropoietin receptor	*EPOR*	3.7
NM_000693	Aldehyde dehydrogenase 1 family, member A3	*ALDH1A3*	3.4
NM_057174	Peroxisomal biogenesis factor 16	*PEX16*	3.1
NM_003784	Homo sapiens serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 7	*SERPINB7*	3.1
NM_002317 [d]	Lysyl oxidase	*LOX*	3.0
AL137663	cDNA DKFZp434G227	*LL5beta*	2.4
NM_001993 [c]	Coagulation factor III (thromboplastin, tissue factor)	*F3*	2.3
U28727	Human pregnancy-associated plasma protein-A preproform	*PAPPA*	2.0
NM_004060	Cyclin G1	*CCNG1*	1.9
NM_004334 [c]	Bone marrow stromal cell antigen 1	*BST1*	1.9
NM_001304	Carboxypeptidase D	*CPD*	1.8
NM_012250 [c]	RAS viral (r-ras) oncogene homolog 2	*RRAS2*	1.7
NM_024109	Hypothetical protein MGC2654	*MGC2654*	1.7
NM_014812	KARP-1-binding protein	*KAB*	1.7
NM_006867	RNA binding protein with multiple splicing	*RBPMS*	1.7
NM_006667 [b,c]	Progesterone receptor membrane component 1	*PGRMC1*	1.6
NM_007223 [b,c]	Putative G protein coupled receptor	*GPR*	1.5
BC001830 [c]	Transforming growth factor beta 1 induced transcript 1	*MGC4078*	1.5
NM_004368 [c]	Calponin 2	*CNN2*	1.5
NM_006270 [c]	Related RAS viral (r-ras) oncogene homolog	*RRAS*	1.5

Genes marked by [a] are genes influenced by culture media differences. Genes belonging to the GO cellular component category integral to plasma membrane are marked by [b], plasma membrane by [c] and extracellular matrix by [d].

Validation of micro-array expression data by real-time RT-PCR

The results from the real-time RT-PCR assays for the genes *VCAM1, KIAA1644, FGFR2, COL27A1*, *IMP-3* and *MME* are presented in Fig. 1. The average fold differences as obtained by real-time RT-PCR were 131.3, 78.2, 78.3, 3.8, 4.2 and 1.5, respectively. The first four genes had a higher expression in osteoblasts, whereas the other two had a higher expression in fibroblasts. Although differences between both cell types were larger with the real-time RT-PCR assays, micro-array analysis results were confirmed. The real-time RT-PCR data (Fig. 1) showed that the transcript abundance between osteoblasts and fibroblasts was significantly different for each of the genes: *VCAM1* (*P* <0.00001), *KIAA1644* (*P* <0.00001), *FGFR2* (*P* <0.00001), *COL27A1* (*P* <0.01), *MME* (*P* <0.00001) and *IMP3* (*P* <0.00001).

Figure 1: Gene expression differences between osteoblasts and fibroblasts in culture. Expression level differences in mRNA between osteoblasts (n = 10) and fibroblasts (n = 11) for the genes *VCAM1, KIAA1644, FGFR2*, *COL27A1, MME* and *IMP3* as determined by real-time RT-PCR are presented as the average fold differences ($2^{-\Delta\Delta Ct}$) ± SEM after normalisation for β2-microglobulin expression levels (** *P* <0.00001; * *P* <0.01).



Gene ontology analysis

Gene ontology classes among the 120 most significantly differentially expressed genes between osteoblasts and fibroblasts were tested for overrepresentation with respect to the final set of 2,643 genes used for significance testing (data not shown). Genes encoding components of the plasma membrane and mediating cell communication and/or cell adhesion and genes encoding proteins with receptor activity were significantly overrepresented in the set of 120 selected genes. Genes that belong to GO categories integral to the plasma membrane, plasma membrane and extracellular matrix have been marked in Table 2.

Discussion

In order to define molecular markers that can be used to discriminate between osteoblasts and fibroblasts we identified osteoblasts grown out of bone fragments in culture medium by micro-array gene expression profiling. We found 120 genes that are significantly differentially expressed between osteoblasts and fibroblasts cultured for only a single passage. Expression of the great majority (97%) of these genes is not seriously influenced by differences between the culture media for both cell types. Using four different class prediction methods, we showed by leave-one-out cross validation that the set of 120 genes predicts the correct class for each sample with high accuracy ($P < 0.0005$). For only a single osteoblast sample the predictive value of one of the methods, the Support Vector Machine Predictor algorithm, appeared to be insufficient. That sample had more missing values for the genes passing the filtering criteria than the other individual samples. Micro-array results for six genes of the 46 most significantly differentially expressed genes were confirmed by real-time RT-PCR. Differences in mRNA expression were larger in the latter assay. In micro-arrays, the relative differences in mRNA expression are often underestimated due to ratio compression. It has been suggested that this ratio compression is caused by a longer time to reach hybridisation equilibrium and certain target and probe features (Dorris et al., 2003).

In an earlier study, Han et al. (2002) performed gene expression profiling of osteoblasts in relation to fibroblasts by cDNA micro-array analysis. They presented a list of also 120 genes with a significantly different expression between osteoblasts and fibroblasts. Only one gene, namely *FOS*, also occurs in our list of 120 such genes. This small overlap may be due to the smaller set of genes analysed by Han et al. (2002): 9,018 versus 21,392 in our case and/or to differences in cell culturing, such as multiple passages in the study of Han et al. (2002) versus a single passage in our study. We have tested by real-time RT-PCR in our osteoblast and fibroblast samples the two markers, *MMP10* and *MFG-E8*, published by Han et al. (2002) as having the largest fold difference amongst protease/inhibitor genes and apoptosis genes respectively. For *MMP10* our results confirmed on average the results found by Han et al. (2002), but comparison of all pairs of individual samples did not show a consistently higher expression of *MMP10* in fibroblasts than in osteoblasts. For *MFG-E8*, we found in contrast to Han et al (2002) no difference in expression between osteoblasts and fibroblasts (data not shown). Therefore, we exclude these two genes as markers to discriminate between osteoblasts and fibroblasts.

In our study, two-thirds of the genes differentially expressed between osteoblasts and fibroblasts have a significantly higher expression in osteoblasts than in fibroblasts. These include *VCAM1, KIAA1644, FGFR2, COL10A1, COL27A1* and *HMOX1*. Among the 120 significantly differentially expressed genes commonly considered as osteoblast-specific markers, such as the genes encoding bone morphogenetic protein (BMP) 2, BMP4, osteocalcin, osteopontin and alkaline phosphatase did not occur. Only for the gene encoding osteopontin (*OPN*), a signal could be detected in the analysed samples. Although on average *OPN* had a twofold higher expression in osteoblasts than in fibroblasts, the high individual variation due to low signal intensities caused exclusion of the gene from the list of genes having a significant differential expression. The other genes that are generally considered as being osteoblast-specific might well become induced in differentiation stages later than those of our early osteoblasts and are, therefore, missing from our analysis.

COL10A1, VCAM1 and *FGFR2* have been described to be involved in osteoblast function. *FGFR2* plays a role during bone formation, with specific involvement in osteoblast replication, differentiation and apoptosis (Marie, 2003). For *COL10A1* and *VCAM1*, relations with the onset of growth and extracellular matrix mineralisation have been described. During bone formation in a mouse model after several days of stimulation with hBMP-2, *COL10A1* has a similar expression profile as *COL2A1*, which is highly expressed during growth and the first part of cell differentiation and *VCAM1* is similar in expression to *Runx2*, which is related to matrix maturation and mineralisation in osteogenesis (Clancy et al., 2003; Stein et al., 2004). Final differentiation of osteoblasts into osteocytes is not expected in primary osteoblast cultures in vitro, but our results show that *COL10A1* and *VCAM1* have already a prominent expression in the osteoblasts and/or that the process of matrix mineralisation has already started in these cells.

We observed an overrepresentation of genes encoding components of the plasma membrane, cell communication, cell adhesion and genes encoding proteins with receptor activity. Several of these genes may be used for identification of osteoblasts in a cell mixture obtained from bone. The list of 120 significantly differentially expressed genes contains many genes such as *VCAM1, KIAA1644, SLC14A1, HLA-DRA* and *MGC14656*, whose protein products can most likely be, used as targets for differential selection markers.

In conclusion, using micro-array-based gene expression profiling we found 42 genes that were significantly differentially expressed between cultured osteoblasts and fibroblasts. For six genes results were confirmed by real-time RT-PCR. Gene

expression analysis of these genes, *VCAM1, KIAA1644, FGFR2, COL27A1, IMP3* and *MME*, showed a consistent difference between individual osteoblast and fibroblast cultures with both micro-array analysis and real-time RT-PCR. This allows for a fast and direct characterisation of small numbers of cells cultured from bone explants.

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Chapter 3

Functional differences between cultured osteoblasts and osteosarcoma cell lines as reflected by gene expression profiles

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Abstract

We have compared expression profiles of cultured primary osteoblasts and osteoblastic osteosarcoma cell lines by micro-array analysis in order to gain more insight into the molecular events underlying the development of osteosarcoma. A significantly different expression was found for 286 genes. For individual genes represented on the array by different oligonucleotides, results from the different pairs were concordant. Two genes with a significantly higher expression in the osteosarcoma cell lines and two genes with a significantly lower expression in the osteosarcoma cell lines were subjected to real-time RT-PCR analysis. The results confirmed those obtained by micro-array analysis. Functional annotation data revealed a lower expression in osteosarcoma cell lines of genes encoding proteins of the cell membrane and extracellular matrix and a higher expression of genes coding for nuclear proteins, such as cell cycle-related and DNA repair-associated proteins. In the osteosarcoma cell lines, an average lower expression was found for the MMP gene cluster at 11q22 and for the KAP gene cluster at 17q21.2. An average higher expression was found for the HOXB cluster at 17q21.3. Such co-expression of genes points to either chromosomal losses in tumours and tumour cell lines or to a common transcriptional regulation. The latter seems the more likely explanation for part of these findings.

Introduction

The progenitor of the most common type of osteosarcoma, osteoblastic osteosarcoma, representing about 40% of all bone tumours (Unni, 1998), is the osteoblast. Osteoblasts normally produce osteoid which by deposition of calcium can be converted into a mineralised hard matrix in which the non-dividing cells remain as osteocytes (Stains and Civitelli, 2003; Harada and Rodan, 2003).

The genetic alterations causing osteosarcoma development or contributing to it are largely unknown, although mutations have been found in several known cancer-related genes, such as *RB1, TP53, CDKN2A, CDKN2B, DCC, CDK4* and *MDM2* (Horstmann et al., 1997; Lohmann and Gallie, 2004; Park et al., 2004). *TP53* and *RB1* are the most frequently mutated genes in tumours in general, including osteosarcoma with percentages of 40% to 60% for *TP53* and 50% to 70% for *RB1* (Hansen et al., 1985; Wadayama et al., 1994; Stock et al., 2000; Sandberg and Bridge, 2003; Overholtzer et al., 2003; van Dartel et al., 2004). Less frequently mutated in osteosarcoma are *CDKN2A* and *CDKN2B* with percentages of 5% to 10% (Miller et al., 1996a;Tsuchiya et al., 2000). Since, these genes have mutations in up to 60% of osteosarcoma cell lines, they probably have a role in tumour cell growth in culture (Miller et al., 1996). *DCC* seems to be frequently mutated in osteosarcoma (50% to 70%), but only a small number of tumours have been analysed (Horstmann et al., 1997). Less frequently in primary osteosarcoma, but more frequently in recurrent or metastatic osteosarcoma, *MDM2* is mutated with frequencies that range from 4% to 27% (Oliner et al., 1992; Park et al., 2004).

Furthermore, cytogenetic analysis and analysis by comparative genomic hybridisation have shown frequent chromosomal involvement of 1p, 2q, 3q, 6p, 8q, 10, 13q, 15q, 17p, 18q and 20 (Squire et al., 2003; Lau et al., 2004; Man et al., 2004), indicating that these chromosomal regions may contain genes involved in the development of osteosarcoma.

We have previously used gene expression profiling of primary unstimulated osteoblasts as a fast and efficient test to characterise these cells and discriminate them from fibroblasts (M. Wilkens, K.A. Kooi, E.A.D. Plantinga, G.J. te Meerman, R.M.W. Hofstra, C.H.C.M. Buys, F. Gerbens, submitted). Here we compare gene expression profiles of cultured osteoblasts with those of osteosarcoma cell lines to gain more insight into which genes play a role in the molecular events leading to osteosarcoma.

Material & Methods

Patient and cell line material

Four females and one male undergoing surgery for a total knee replacement at ages ranging from 57 to 84 were included in this study as donors of trabecular bone fragments. All samples were collected fully anonymously and used in accordance with the ethical regulations of the hospital.
Five human osteoblastic osteocarcoma cell lines namely MG63 (ATCC CRL-1427), SaOS-2 (ATCC-HTB-85), HOS (ATCC CRL-1543), U-2 OS (ATCC-HTB-96) and G292 (ATCC-CRL-1423) obtained from the American Type Culture Collection (ATCC, Baltimore, MD, USA) were included in this study.

Culturing of primary osteoblasts and osteosarcoma cell lines

Trabecular bone fragments were treated as described by Scheven et al. (1991) to obtain primary osteoblastic cells. Specimens were transported under sterile conditions from the operating centre to the laboratory, chopped into pieces of 0.2 cm – 1.0 cm in diameter and washed three times with sterile phosphate-buffered saline. Bone fragments were treated with collagenase (1 mg/ml) (GIBCO - Invitrogen Inc, Carlsbad, Ca, USA) at 37°C during 2.5 h and again washed three times with sterile phosphate-buffered saline. Subsequently, the bone fragments were cultured in Dulbecco‘s modified Eagle medium (DMEM) with l-glutamax (GIBCO - Invitrogen) supplemented with 100 IU/ml penicillin and 1000 µg/ml streptomycin (all from GIBCO - Invitrogen) and 10% foetal bovine serum (Biowhittaker – Cambrex, Verviers, Belgium) in small culture flasks (25 cm^2) at 37°C in a humidified atmosphere with 5% CO_2. Cells isolated as osteoblasts were harvested for RNA isolation after reaching 70% - 80% of confluence by trypsin (0.05%)/EDTA (0.02%) (GIBCO – Invitrogen) treatment, pelleted, washed with sterile phosphate-buffered saline, pelleted again and stored at –80°C until RNA isolation. Characterisation of the isolated osteoblasts was performed by comparison to fibroblasts through real-time RT-PCR analysis of six tissue-specific genes (M. Wilkens, K.A. Kooi, E.A.D. Plantinga, G.J. te Meerman, R.M.W. Hofstra, C.H.C.M. Buys, F. Gerbens, submitted).
The human osteoblastic osteosarcoma cell lines MG63, SaOS-2 and HOS were cultured on RPMI1640 (GIBCO – Invitrogen), while U-2 OS and G292 were grown on

McCoy's 5A (GIBCO - Invitrogen). Both media were supplemented with 10% foetal bovine serum (Biowhittaker), 2 mM glutamine, 100 U/ml penicillin, 1000 μg/ml streptomycin and 250 μg/ml fungizone amphotericine B (all from GIBCO - Invitrogen). All cell lines were cultured in culture flasks at 37°C in a humidified atmosphere with 5% CO_2. After having reached 70% to 80% of confluence, human osteosarcoma cells were harvested by trypsin (0.05%)/EDTA (0.02%) (GIBCO - Invitrogen) treatment, pelleted, washed with sterile phosphate-buffered saline, pelleted again and stored at –80°C until RNA isolation.

RNA isolation

Total RNA was isolated with the RNeasy mini kit (Qiagen, Valencia, CA, USA) and subsequently treated with DNase I (Qiagen) as described by the manufacturer. Total RNA yield and purity were calculated by measuring absorbances at 260 nm and 280 nm spectrophotometrically on a NanoDrop® ND-1000 UV-VIS Spectrophotometer (Nanodrop technologies, Wilmington, DE, USA). RNA integrity was determined by 1% formaldehyde agarose gel electrophoresis.

mRNA Amplification and Cy-dye coupling

Linear amplification of mRNA was performed essentially according to a protocol of the Dutch Cancer Institute (www.nki.l.nl/nkidep/pa/microarray/protocols.htm). Briefly, amplification started with first strand cDNA synthesis from 2 μg of total RNA, using Superscript II RT-polymerase (GIBCO - Invitrogen) and a specific oligo(dT) primer containing a 17bp T7 polymerase recognition site
(5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG$[T]_{24}$-3')
(Eurogentec, Seraing, Belgium). After second strand synthesis, double-stranded cDNA was purified with the Qiaquick PCR purification kit (Qiagen) and the yield was determined spectrophotometrically. In vitro transcription was performed with the T7 Megascript kit (Ambion, Huntingdon-Cambridgeshire, UK) as described by the manufacturer, but using instead of UTP a 1:1 mixture of aminoallyl-UTP (Ambion) and UTP with a final concentration of 7.5 mM for all NTPs ('t Hoen et al., 2003). Amplified RNA (aRNA) was purified with the RNA clean up protocol (Qiagen). Five μg of aRNA was labelled by coupling monoreactive Cyanine 3 (2.5 nmol per reaction) or Cyanine 5 (2.5 nmol per reaction) fluorophores (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) to the aminoallyl-modified nucleotides. Labelled

aRNA was separated from unincorporated Cyanine 3 or Cyanine 5 molecules with Microspin G50 columns (Amersham) as described by the manufacturer.

Experimental design

The five primary osteoblast cultures and the five osteoblastic osteosarcoma cell lines were analysed as ten independent samples. Each sample was labelled with Cyanine-3 and Cyanine-5 separately and subsequently assigned at random to a sample of the opposite colour for hybridisation.

Micro-array Slides & Hybridisation

In-house manufactured human oligonucleotide arrays were used containing the Qiagen/Operon 21,329 70-mer human gene specific oligonucleotide set version 2.1 extended with 4,000 negative and positive control features. The oligonucleotides were printed in a concentration of 10 pM on Ultra-GAPS amino-silane coated slides (Corning BV. Life Sciences, New York, USA) using BioRobotics 10K quill pins with the MicroGrid spotter (Isogen, Maarssen, The Netherlands). Blocking, prehybridisation and hybridisation were performed as described by Hegde et al (2000), with some modifications. In short, slides were blocked with ethanolamine at 52°C during 1 h. Prehybridisation was done with prewarmed prehybridisation buffer containing 0.5% bovine serum albumin (Sigma - Aldrich, St. Louis, MO, USA) at 52°C during 45 min. Subsequently, the slides were washed 6 times with preheated water (52°C), dried by centrifugation at 800 rpm during 3 min and immediately used for hybridisation. The hybridisation sample consisted of the fluorescently labelled probe mixture and 30 µg poly-A (Sigma - Aldrich) mixed with an equal volume of preheated (52°C) 2X hybridisation buffer. This hybridisation sample was heated at 95°C for 3 min before it was applied to the preheated slides. Hybridisation was performed in hybridisation chambers (Telechem International Inc, Sunnyvale, CA, USA) in a waterbath at 52°C in the dark for approximately 48 h. Subsequently, slides were washed with 5 wash solutions under agitation: 1xSSC/0.2% SDS at 52ºC; 0.1xSSC/0.2% SDS at 52ºC; 0.1xSSC at 52ºC; 0.1xSSC at RT and 0.01xSSC at RT. Each wash step lasted 5 min. Finally slides were dried by centrifugation at 800 rpm during 3 min and scanned with an Affymetrix GMS428^{TM} array scanner.

Micro-array data analysis

Fluorescent signal intensity data for each spot and for each fluorophore were extracted from the scanned images of each micro-array slide using ImaGene version 5.6 (BioDiscovery, El Segundo, California, USA). Signal intensity data were log transformed and for each spot the Cyanine-5 signal intensity/Cyanine-3 signal intensity ratio was determined and subjected to print-tip loess intensity dependent normalisation using the Limma package from the Bioconductor project in R (http://bioinf.wehi.edu.au/limma). Since no dependency exists between both samples during hybridisation ('t Hoen et al., 2004), normalised log-ratios were back transformed to log intensities. Further data analysis was performed using BRB ArrayTools v3.2 developed by Dr. Richard Simon and Amy Peng Lam (http://linus.nci.nih.gov/~brb/download.html). Basically, data was vigorously filtered to exclude control spots, empty spots, spots with high between-pixel-intensity variability and spots designated as bad by eye. Genes that had more than 25% missing data across all observations were excluded from the analysis. Genes significantly differentially expressed between the primary osteoblast cultures, and the osteosarcoma cell lines were identified by an F-test, using a randomised variance model and accounting for replicate readings of the same sample. Moreover, a multivariate permutation test (Reiner et al., 2003) was applied to account for a false discovery rate of only 10% in the set of significantly differentially expressed genes. Genes retained after filtering were hierarchically clustered by comparing their expression profiles across all samples by average linkage clustering. The robustness of the clustering results was tested by applying 100 independent perturbations of the data and re-clustering (McShane et al., 2002).

For each osteosarcoma cell line, chromosomal regions with correlated gene expression were identified by CGH-miner (http://www-stat.stanford.edu/~wp57/CGH-Miner). Therefore, for each gene the signal intensity of each osteosarcoma cell line compared to the mean signal intensity of all primary osteoblast cultures was calculated and smoothed (moving average window of 5 genes) before the clustering along the individual chromosomes was applied. To determine whether chromosomal regions have similar co-expression across all osteosarcoma cell line samples, a false discovery rate of 1% was applied when compared to data of normal versus normal arrays (Wang et al., 2005).

Overrepresentation of the selected genes in gene ontology categories (GO; http://www.geneontology.org) as compared to the entire filtered gene list was

analysed by EASE/DAVID version 2.0 (http://david.niaid.nih.gov/david/ease.html). Significant GO categories were identified based on the EASE score after Bonferroni correction for multiple testing (Hosack et al., 2003). Further functional analysis was performed by Pathway Assist (http://www.ariadnegenomics.com/products/pathway.html) through building and visualizing gene networks from our selected set of genes, based on a wide variety of biological data as collected with the automated text-mining program MedScan from PubMed and other public databases.

Real-time RT-PCR

For real-time Reverse Trancsriptase PCR (real-time RT-PCR), cDNA was generated by using Ready-To-Go™ You-Primed First-Strand Beads (Amersham Biosciences) and an oligo(dT) primer (Eurogentec) as described by the manufacturer. Primers were designed with the programme Primer Express (Applied Biosystems, Foster City, California, USA). These are shown in Table 1. Each RNA sample was analysed in triplicate in a final volume of 20 μl in a 384-well plate with the ABI Prism 7900 Sequence Detection System (Applied Biosystems). Real-time RT-PCR data was analysed with the Sequence Detection System software (SDS 2.1.1, Applied Biosystems). For each gene the threshold cycle value (Ct) of every sample was extracted from the amplification plots. The difference in expression between osteosarcoma cell line samples and the primary osteoblast samples was determined for each gene using the ΔΔ median threshold cycle number (Ct) method. The corresponding fold change in expression was calculated by the formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).
Real-time RT-PCR data were statistically analysed using analysis of variance (ANOVA), including the β_2-microglobulin gene as a covariate in the model.

Table 1: Real-time RT-PCR primer sequences.

Gene	*Forward primer*	*Reverse primer*	*Product size*
B2M	TGACTTTGTCACAGCCCAAGATA	AATGCGGCATCTTCAAACCT	79 bp
WISP2	CACACAGCCTATATCAAACATGCA	TAAGGGACTGCTTGTCCCATCT	83 bp
PRIM1	GAAGAGCCCTTTTAGTGTTCATCCT	AATGGATCAAACTGGTCCACTTTC	84 bp
VCAM1	CATGGAATTCGAACCCAAACA	GCTGACCAAGACGGTTGTATCTC	85 bp
COL4A5	ACAGCTTTTGGCTGGCAACT	CGGCTAATTCGTGTCCTCAAG	97 bp

Results

Expression profiling of primary osteoblasts and osteosarcoma cell lines

Using 21K oligonucleotide micro-arrays, we performed gene expression profiling of cultures of five primary osteoblast isolates and five osteoblastic osteosarcoma cell lines in a randomised model. After filtering and normalisation the 10 samples were hierarchically clustered based on the transcriptional profile of the remaining 7,397 genes. This showed a clear and consistent distinction between both classes of samples (Fig. 1). The dendrogram also shows that the variation between replicates of the same sample is less than the variation between different samples.

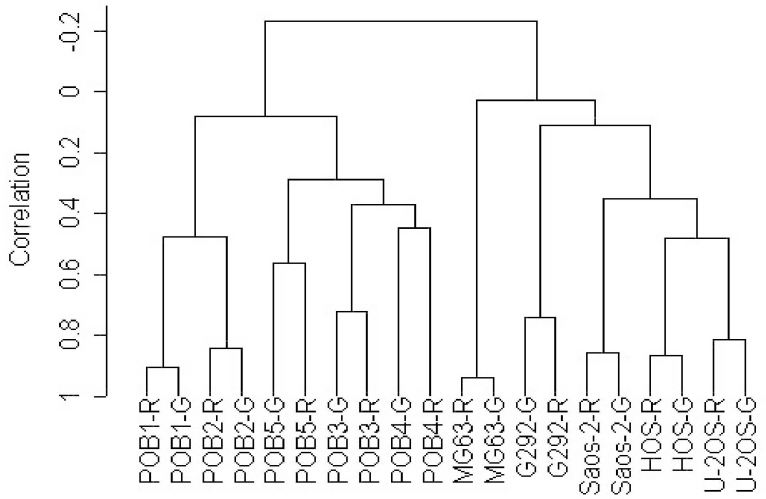

Figure 1: Hierarchical clustering tree of the expression profiles of 7,397 genes for cultures of five primary osteoblast specimens (POB) and of five different osteosarcoma cell lines. Each sample has been hybridised twice using different Cyanine fluorophores, G (Cy3) and R (Cy5). Correlations between the expression profiles of the samples are indicated by the height of the common knot in the tree.

Two hundred and eighty six genes were significantly ($P < 0.001$) differentially expressed between the cultures of the primary osteoblast isolates and of the osteoblastic osteosarcoma cell lines. The 100 most significant differences represented 97 different genes of which 20 genes had a 2.8 to 6.8 fold overexpression in the osteosarcoma cell lines and 77 genes were higher expressed in primary osteoblasts with fold differences ranging from 2.7 to 66.7. The 10 most significantly higher expressed or lower expressed genes for the two classes, respectively, are listed in Table 2.

Table 2: Ten most significantly differentially expressed genes in cultures of five osteosarcoma cell lines compared to cultures of five primary osteoblast specimens.

GenBank Accession	*Gene Symbol*	*Gene Coding for*	*Fold Difference*
Higher expressed			
AK026894	*RHPN2*	Rhopilin, Rho GTPase binding protein 2	6.8
NM_033380	*COL4A5*	Collagen, type IV, alpha 5 (Alport syndrome)	6.2
NM_000946	*PRIM1*	Primase, polypeptide 1, 49kDa	5.6
NM_024094	*DCC1*	Defective in sister chromatid cohesion homolog 1 (S. cerevisiae)	5.2
BC010437	*MGC22679*	Hypothetical protein MGC22679	5.2
NM_012198	*GCA*	Grancalcin, EF-hand calcium binding protein	5.0
NM_016623	*FAM49B*	Family with sequence similarity 49, member B	5.0
NM_014791	*MELK*	Maternal embryonic leucine zipper kinase	4.3
NM_004111	*FEN1*	Flap structure-specific endonuclease 1	4.2
NM_014176	*HSPC150*	HSPC150 protein similar to ubiquitin-conjugating enzyme	4.2
Lower expressed			
NM_015429	*ABI3BP*	ABI gene family, member 3 (NESH) binding protein	66.7
NM_001078	*VCAM1*	Vascular cell adhesion molecule 1	47.6
NM_003881	*WISP2*	WNT1 inducible signalling pathway protein 2	41.7
NM_007281	*SCRG1*	Scrapie responsive protein 1	32.3
NM_001353	*AKR1C1*	Aldo-keto reductase family 1, member C1	32.3
NM_006475	*POSTN*	Periostin, osteoblast-specific factor	26.3
NM_032849	*FLJ14834*	Hypothetical protein FLJ14834	17.5
NM_022842	*CDCP1*	CUB domain-containing protein 1	14.4
NM_002427	*MMP13*	Matrix metalloproteinase 13 (collagenase 3)	13.9
AK021531	*COL3A1*	Collagen, type III, alpha 1	13.2

The selected genes are listed according to fold difference.

Several of the 286 genes are represented in the gene list by two independent oligonucleotides. Results for these pairs (Table 3) were concordant.

Table 3: Consistency of differential gene expression as assessed by different oligonucleotides representing the same gene.

Gene coding for	GenBank Accession	Rank *	Fold Difference **
Forkhead box C1	NM_001453	12	0.1
	AK021858	13	0.1
Phosphatase, orphan 2	BC010437	16	5.2
	BC016950	31	5.0
Collagen type VIII, alpha 1	AL359062	22	0.1
	NM_001850	23	0.1
ARG99 protein	BC017984	123	0.2
	AK055962	229	0.4
UDP-N-acetyl-alpha-D-galatosamine	AK024931	248	0.4
	AK023815	265	0.4
Dystonin	AB018271	211	0.4
	BC016991	239	0.2
	NM_020388	NS	0.8
Chromosome 5 open reading frame 13	AK021624	38	0.3
	NM_004772	NS	0.4
Lamin B2	M94362	58	2.2
	NM_032737	NS	2.1
Epithelial membrane protein 1	BC017854	89	0.1
	NM_001423	NS	0.2
ATPase, Class I, type 8B, member 2	AB032963	177	0.3
	AK054886	NS	0.7

* Rank is the ranking in the complete list of 286 significantly differentially expressed genes;
NS indicates non-significant, meaning that the oligonucleotide does not occur in the list of 286 significantly differentially expressed genes.
** Fold difference reflects the expression in osteosarcoma cell lines as compared to primary osteoblasts.

Validation of micro-array expression data by real-time RT-PCR

Two genes, *COL4A5* and *PRIM1*, with a significantly higher expression in the osteosarcoma cell lines and two genes, *VCAM1* and *WISP2*, with a significantly lower expression in the osteosarcoma cell lines, were assessed by real-time RT-PCR. After analysis of the PCR products in a 3% agarose gel, it was only in one cell line, SaOS-2, that we did not observe a PCR product for one of these four genes, namely *COL4A5*. Therefore, the average level of mRNA expression of *COL4A5* has been calculated over four osteosarcoma cell lines instead of five. *COL4A5* and *PRIM1* were significantly higher expressed in the osteosarcoma cell lines with average fold changes of 28.2 ($P < 0.05$) and 9.6 ($P < 0.00001$), respectively. *VCAM1* and *WISP2* were significantly ($P < 0.00001$) and very strongly lower expressed in osteosarcoma cell lines with average fold changes of 106.0 and 217.2, respectively (Fig. 2).

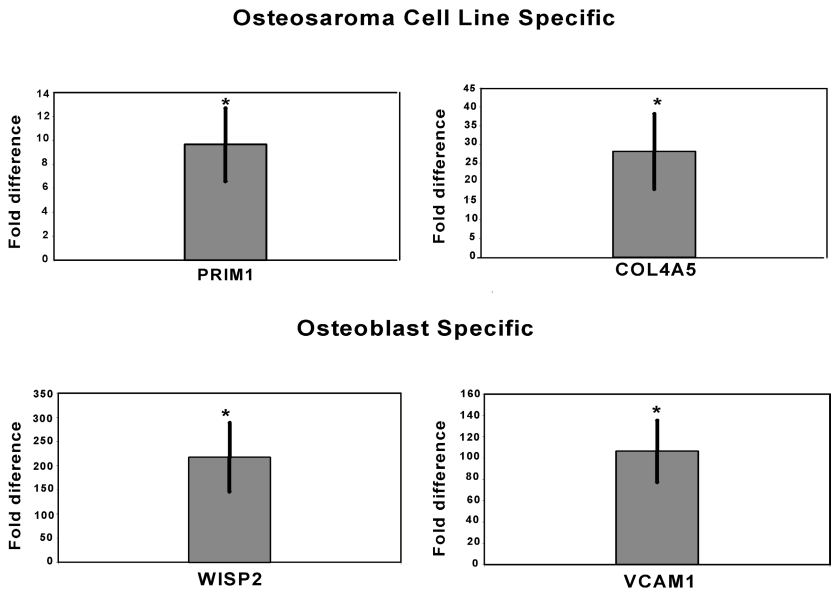

Figure 2: Gene expression differences between osteosarcoma cell lines and primary osteoblasts in culture**.** Expression level differences in mRNA between osteosarcoma cell lines (n = 5) and osteoblast isolates (n = 5) for the genes *PRIM1, COL4A5, WISP2* and *VCAM1* as determined by real-time RT-PCR are presented as the average fold difference ($2^{-\Delta\Delta Ct}$) ± SEM after normalisation for β2-microglobulin expression levels (* P < 0.00001).

Functional analysis

Amongst the 286 differentially expressed genes, 184 genes had a functional annotation. Analysis of these 184 genes showed that genes encoding proteins associated with the cell membrane or the extracellular domain have generally a lower expression in osteosarcoma cell lines than in cultured osteoblasts. Genes coding for nuclear proteins are generally higher expressed in osteosarcoma cell lines (Table 4).

Among the 97 most significantly differentially expressed genes, gene ontology (GO) annotation analysis showed a strong overrepresentation of genes belonging to the GO classes catalytic activity, binding, cell communication, cell adhesion and extracellular domain (data not shown).

Table 4: Functional classification of 184 genes with an annotation among the 286 differentially expressed genes.

A: Genes higher expressed in osteosarcoma cell lines

GenBank Accession	*Gene Symbol*	*Fold Difference*	*GenBank Accession*	*Gene Symbol*	*Fold Difference*	*GenBank Accession*	*Gene Symbol*	*Fold Difference*
Extracellular matrix			**Membrane**			**Nucleus**		
NM_001333	*CTSL2*	7.7	NM_012198	*GCA*	5.0	NM_004411	*FEN1*	4.2
			AK021664	*SLC24A5*	4.0	NM_004153	*ORCIL*	3.8
			NM_004233	*CD83*	2.7	NM_018365	*MNS1*	2.9
			AB006624	*KIAA0286*	2.4	NM_024680	*FLJ23311*	3.7
						NM_021927	*FLJ13220*	2.9
						NM_002358	*MAD2L*	7.1
						NM_014708	*KNTC1*	2.1
						NM_022909	*CENPH1*	4.8
						NM_004502	*CDCA5*	3.8
						NM_001730	*KLF5*	5.6
						NM_000946	*PRIM1*	5.6
						NM_017955	*CDCA4*	3.1
						NM_003090	*SNRPA1*	3.8
						NM_000251	*MSH2*	4.5
						NM_002266	*KPNA1*	3.1

B. Genes lower expressed in the osteosarcoma cell lines

GenBank Accession	*Gene Symbol*	*Fold Difference*	*GenBank Accession*	*Gene Symbol*	*Fold Difference*	*GenBank Accession*	*Gene Symbol*	*Fold Difference*
Extracellular matrix			**Membrane**			**Nucleus**		
NM_001393	*ECM2*	6.4	AF380356	*XG*	5.9	X68742	*PELO*	4.5
NM_014467	*SRPX2*	5.3	NM_002349	*Ly75*	5.8	AK027738	*FOXP1*	3.2
NM_015424	*CHL2*	5.7	AK002171	*FLJ11309*	2.7	NM_119025	*SMOX*	3.3
NM_000062	*SERPING1*	7.5	AB032963	*ATP8B2*	2.9	NM_021972	*SPHK1*	4.7
AB021124	*CHST2*	5.6	BC017854	*EMP1*	12.3	AK021858	*FOXC1*	7.2
NM_000396	*CTSK*	10.1	NM_005279	*F2R*	4.8	NM_005982	*SIX1*	3.3
NM_007281	*SCRG1*	32.3	NM_002231	*KAI1*	5.1			
NM_000362	*TIMP3*	5.6	NM_031866	*FZD8*	4.5			
NM_006475	*POSTN*	26.3	NM_001078	*VCAM1*	47.6			
NM_003278	*TNA*	7.1	NM_014556	*EVC*	4.3			
NM_007036	*ESM1*	7.6	NM_054027	*ANKH*	4.3			
NM_001734	*C1S*	6.9	AF010236	*SGCD*	5.6			
NM_002009	*FGF7*	3.9	NM_033518	*SLC38A5*	5.3			
NM_000095	*COMP*	12.5	NM_007361	*NID2*	10.0			
NM_004750	*CRLF1*	12.4	NM_032812	*PLXDC2*	6.4			
NM_002402	*MEST*	11.9	AB006624	*KIAA0286*	0.4			
NM_031476	*DKFZP434B044*	4.8	NM_004791	*ITGBL1*	8.5			
NM_000609	*CXCL12*	4.2	NM_001257	*P105*	5.0			

Chromosomal regions with correlated expression

In the individual osteosarcoma cell lines, several genomic regions showed co-expression of genes (data not shown). We identified 7 regions in which genes, belonging to the 7,397 genes remaining after filtering, showed correlation in expression over all five osteosarcoma cell lines, i.e. 5q15, 9q22-q22.3, 11q22.2, 17q21.2, 17q21.3, 17q24.2 and 20q12-q13.1. One of these regions, 17q21.3, showed an increased average expression of their genes, whereas the other chromosomal regions showed a decreased average expression of their genes in each of the osteosarcoma cell lines (Table 5).

Table 5: Genomic regions with correlated gene expression changes across the osteosarcoma cell lines HOS, SaOS-2, MG63, U-2 OS and G292 with the genes strongly contributing to the regional co-expression.

Chromosomal Region	*GenBank Accession*	*Gene Symbol*	*Gene coding for*	*Average Expression*
5q15	NM_000439	*PCSK1*	Proprotein convertase subtilisin/kexin type 1	Lower
	NM_001750	*CAST*	Calpastatin	
9q22-q22.3	NM_017948	*NOL8*	Nucleolar protein 8 ,	Lower
	NM_033014	*OGN*	Osteoglycin (osteoinductive factor, mimecan)	
	NM_005014	*OMD*	Osteomodulin,	
	NM_017680	*ASPN*	Asporin (LRR class 1)	
	NM_001393	*ECM2*	Extracellular matrix protein 2	
	AK024267	*FLJ1420*	-	
	AB014599	*KIAA0699*	-	
11q22.2-q22.3	NM_002424	*MMP8*	Matrix metalloproteinase 8 (neutrophil collagenase)	Lower
	NM_002425	*MMP10*	Matrix metalloproteinase 10 (stromelysin 2)	
	NM_002421	*MMP1*	Matrix metalloproteinase 1 (interstitial collagenase)	
	NM_002422	*MMP3*	Matrix metalloproteinase 3 (stromelysin 1, progelatinase)	
	NM_002426	*MMP12*	Matrix metalloproteinase 12 (macrophage elastase)	
	NM_002427	*MMP13*	Matrix metalloproteinase 13 (collagenase 3)	
	NM_025208	*PDGFD*	Platelet derived growth factor D	
17q21.2	NM_031959	*KRTAP3-2*	Keratin associated protein 3-2	Lower
	NM_031957	*KRTAP1-5*	Keratin associated protein 1-5	
	NM_030966	*KRTAP1-3*	Keratin associated protein 1-3	
	NM_030967	*KRTAP1-1*	Keratin associated protein 1-1	
	BC012486	*KRTAP2-1*	Keratin associated protein 2-1	
17q21.3	NM_002145	*HOXB2*	Homeo box B2	Higher
	AL137449	*HOXB4*	Homeo box B4	
	NM_002147	*HOXB5*	Homeo box B5	
	NM_018952	*HOXB6*	Homeo box B6	
	NM_004501	*HOXB7*	Homeo box B7	
17q24.2	NM_004694	*SLC16A6*	Solute carrier family 16 (monocarboxylic acid transporters), member 6	Lower
	AK056789	*FAM20A*	Family with sequence similarity 20, member A	
	NM_007168	*ABCA8*	ATP-binding cassette, subfamily A (ABC1), member 8	
20q12-q13.1	NM_003881	*WISP2*	WNT1 inducible signalling pathway protein 2	Lower
	NM_022358	*KCNK15*	Potassium channel, subfamily K, member 15	

Discussion

To increase our understanding of the molecular events that underlie osteosarcoma development, we wanted to identify genes differentially expressed between osteosarcoma and its progenitor cell, the osteoblast, by global gene expression profiling. In our study, osteosarcoma is represented by five independently established cell lines. As osteoblasts we used five different isolates from bone fragments, cultured for a single passage without any additional specific stimulation. The cells from these isolates were characterised as osteoblasts and could be clearly discriminated from fibroblasts, based on the expression profiles of six selected genes (M. Wilkens, K.A. Kooi, E.A.D. Plantinga, G.J. te Meerman, R.M.W. Hofstra, C.H.C.M. Buys, F. Gerbens, submitted). We expect that those genes that are differentially expressed between all five osteoblastic osteosarcoma cell lines and all five cultures of primary osteoblasts will include the genes involved in osteosarcoma development.

Hierarchical clustering of vigorously filtered gene expression data showed a clear distinction between the expression profiles of the osteosarcoma cell lines and the cultured primary osteoblasts (Fig. 1). The distinction in gene expression between both sample groups appeared to be mainly caused by a set of 286 significantly differentially expressed genes. Micro-array based results were confirmed internally by results from different oligonucleotides on the array representing the same gene (Table 3) and by real-time RT-PCR results for *PRIM1, COL4A5, WISP2* and *VCAM1* (Fig. 2). The observed differences between real-time RT-PCR and micro-array results for the genes *WISP2* and *VCAM1* are explained by pixel saturation of the spots on the micro-array resulting in an underestimation of the respective fold differences.

For several genomic regions we found a higher or lower average expression of the genes in them, which may suggest that these regions are either affected by loss or gain of chromosomal regions or are subject to a common transcriptional regulation. Ozaki et al. (2003) have reported results of CGH analyses for four of our five osteosarcoma cell lines. Our findings did not show any clear correlation with those results. Analysis of chromosomal regions of co-expression for each cell line separately also did not show any association with the genomic aberrations reported by Ozaki et al. (2003) (data not shown). Therefore, common transcriptional regulation seems a likely explanation for at least part of our findings.

Functional annotation based on MedScan (Pathway assist) was available for 184 of the 286 significantly differentially expressed genes. Many of these genes, encoding

proteins associated with the (glomerular) basement membrane and extracellular matrix, had a lower expression in the osteosarcoma cell lines, e.g. *COL3A1*, *COL5A1*, and *COL8A1* encoding collagens; *LAMA4* encoding laminin; *NID2* encoding nidogen; *THBS1* and *THBS3* encoding thrombospondins; *CDH13* and *PCDH18* encoding cadherins; and *ITGBL1* and *ITGA5* encoding integrins. *MMP1, MMP3, MMP13* and *MMP14*, encoding matrix metalloproteinases, also had a lower expression in the osteosarcoma cell lines than in the cultured osteoblasts. Remarkably, *TIMP3* encoding the MMP inhibitor was also lower expressed. The MMP gene cluster at 11q22.2-11q22.3 exhibited a strongly reduced average expression in all our osteosarcoma cell lines. This type of co-expression of MMP cluster genes has also been observed in normal breast tissue and breast tumours in contrast to several other tissues (Caron et al., 2001). Such tissue-specific co-expression is another argument for common transcriptional regulation. Several other genes involved in maintaining the stability and organization of the extracellular matrix, such as *TNFAIP6* had a lower expression in the osteosarcoma cell lines than in the osteoblast cultures. *KRTAP1-1, KRTAP1-3, KRTAP1-5, KRTHA4* and *KRT19*, genes from the keratin-associated protein (KAP) gene cluster at 17q21.2, had also a lower expression in our osteosarcoma cell lines. This finding may be linked to the keratinocyte growth factor (*FGF7*) expression, which is significantly lower in the cell lines than in the cultured osteoblasts. Co-expression of this KAP cluster-containing region was also found in a large study of 130 breast carcinomas (Reyal et al., 2005). In general, results indicate that the basement membrane and extracellular matrix are less well-defined and organised in osteosarcoma cell lines than in osteoblasts. This has also been reported by others (Vaes et al., 2002; Thomas et al., 2004; Eppert et al., 2005) and may reflect the dedifferentiated state of osteosarcoma tumours and tumourigenic cells in general (Sato et al., 2005). Alternatively, it can reflect the altered environment for cells growing in vitro in specific cell culture media (Sandberg and Ernberg, 2005).

Genes mainly associated with the cell nucleus and involved in cell cycle regulation and cell division, such as *CDC7, CDC25C, CDCA8, PRIM1, RFC4, NUSAP1, HMGN3, RPA2, ECT2, PLK1, KIF22* and *TOP2A*, had a higher expression in the osteosarcoma cell lines, suggesting a higher proliferative activity of these cell lines. In addition, several genes involved in the initiation of replication (*ORC1L, FOXC1*), the regulation of the initiation of transcription (*YEATS4, KLF5*) and the mitotic spindle assembly checkpoint (*MAD2L1, TTK, KIF2B, SPAG5*) were higher expressed in the osteosarcoma cell lines. This may reflect an intrinsic tumour feature or may be the

result of positive selection during cell line establishment of cells with an enhanced proliferation capacity. In this respect, it may be noted that amplification of *PRIM1*, which gene initiates or primes DNA synthesis, has been found in 9 of 22 osteosarcoma specimens tested (Yotov et al., 1999) and that *PRIM1* maps close to *CDK4*, for which amplification has also frequently been found in osteosarcoma (Ladanyi et al., 1993a; Tarkkanen et al., 1995; Ragazzini et al., 1999). The cell division genes *CDC7* and *CDCA8* are both located at the short arm of chromosome 1, an often amplified region in osteosarcoma (Mertens et al., 1993; Fletcher et al., 1994; Zielenska et al., 2001; Ozaki et al., 2003; Atiye et al., 2005). Based on the analysis of chromosomal regions affected by gain or loss of expression, we found one cluster with a higher expression in the osteosarcoma cell lines, namely the HOXB gene cluster at 17q21.3. The core *HOXB5, HOXB6* and *HOXB7* genes and the flanking members had a higher expression in our osteosarcoma cell lines. In breast carcinoma cell lines as well as in tumours, the HOXB cluster has been identified as an amplicon with increases in gene expression related to the copy number (Hyman et al., 2002; Pollack et al., 2002; Reyal et al., 2005). In the osteosarcoma cell lines U-2 OS and SaOS-2, high-level gains were observed for the chromosomal region 17q22~qter by conventional-CGH analysis (Ozaki et al., 2003). The region 17q21.3 flanks 17q22-qter and might not be distinguishable from this latter region by conventional-CGH.

Expression of several DNA repair-associated genes, namely *BRCA2, EXO1, BARD1, KUB3, BLM, MSH2* and *MSH6*, is more prominent in the osteosarcoma cell lines than in primary cultured osteoblasts, suggesting an intensive DNA repair activity of the osteosarcoma cell lines, possibly associated with the higher proliferation. For cell lines in general, a higher expression of DNA repair-associated genes has also been described by Sandberg and Ernberg (2005).

We found that a number of genes involved in the regulation of apoptosis or programmed cell death (*CASP8, CASP9, TNFRS1B, FAF1, TRAF1* and *TRIP*) are affected in their expression. In contrast to our expectation that cell lines would have a repressed apoptotic programme, all pro-apoptotic proteins appeared to have an induced expression, whereas anti-apoptotic proteins were repressed in our osteosarcoma cell lines. *CASP9*, however, supposed to be involved in the caspase activation cascade, has a lower expression in osteosarcoma cell lines, thereby possibly dysregulating programmed cell death (Cardone et al., 1998). Each of the genomic regions for which we found correlated expression patterns have also been found in earlier studies on transcriptome analysis mainly focusing on breast cancer

tumours and cell lines (Caron et al., 2001; Hyman et al., 2002; Pollack et al., 2002; Reyal et al., 2005). Our results indicate that a similar co-expression of genes in these regions also occurs in osteosarcoma cell lines.
An answer to the question whether the lower expression in osteosarcoma cell lines than in osteoblasts of genes encoding membrane-associated proteins and the higher expression of genes encoding nucleus-associated proteins should be considered as cell line-specific or tumour-specific, requires additional comparisons of gene expression profiles of primary osteosarcoma tumours with the profiles from the present study.

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Chapter 4

Gene expression patterns and copy number changes in primary osteosarcoma

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In preparation

Abstract

Expression profiles of six primary osteosarcoma tumours (three fibroblastic and three osteoblastic) were compared to those of five osteosarcoma cell lines and five cultured osteoblasts using micro-array analysis. In total, 838 genes were significantly differentially expressed between any two of these groups of specimens. Functional, primary osteosarcoma tumours were characterised by elevated expression of genes encoding proteins involved in inflammatory processes and angiogenesis when compared to the osteoblast cells and repressed expression of genes associated with transcriptional and translational machinery when compared to the osteosarcoma cell lines. Genes encoding cell cycle regulation-associated proteins were expressed most prominently in the osteosarcoma cell lines. In both the osteosarcoma tumours and cell lines genes belonging to the MMP gene cluster at 11q22 had repressed expression. Furthermore, we have performed array-CGH of the osteosarcoma tumours and compared their DNA content with normal human genomic DNA. We found under-representation of 13q in five of the six tumours. Only in the three osteoblastic tumours under-representation of 11p was found. Under-representation of 2p and 8p appeared to be specific for fibroblastic cases. Amplifications were detected in 13 different chromosomal regions, namely at 6p21, 6pter, 7p, 8q, 9p, 11q, 12p, 12q13, 12q13-q14, 12q14-q21.3, 16q22.1-22.2, 17p and 17q. Gene expression analysis of these amplified regions, based on a comparison with cultured osteoblasts, revealed several new potentially osteosarcoma susceptibility genes and one already known gene (*CDK4*).

Introduction

Conventional osteosarcoma, the most common type of osteosarcoma, is a primary intramedullary high-grade sarcoma that represents about 75% of all bone tumours (Mertens et al., 1993; Spina et al., 1998). The World Health Organisation distinguishes three major subtypes of conventional osteosarcoma: 50% are osteoblastic, 17% fibroblastic and 33% chondroblastic (Unni, 1998; Marina et al., 2004). Osteoblastic osteosarcoma has osteoid or bone as the predominant type of matrix. Fibroblastic osteosarcoma is composed of malignant spindle-forming cells with a slight presence of osteoid (Unni, 1998; Spina et al., 1998; Marina et al., 2004). The progenitor cell of osteosarcoma is the osteoblast. The genetic alterations causing osteosarcoma development or contributing to it are largely unknown, although mutations have been found in several known cancer related genes, such as *RB1, TP53, CDKN2A, CDKN2B, DCC, CDK4* and *MDM2* (Horstmann et al., 1997; Lohmann and Gallie, 2004; Park et al., 2004).

The very complex cytogenetics of primary osteosarcoma makes it difficult to identify chromosomal aberrations specific for osteosarcoma. Cytogenetic analysis and analysis by comparative genomic hybridisation have shown frequent chromosomal involvement of 1p, 2q, 3q, 6p, 8q, 10, 13q, 15q, 17p, 18q and 20 (Zielenska et al., 2001; Squire et al., 2003; Ozaki et al., 2003; Lau et al., 2004; Zielenska et al., 2004; Man et al., 2004; Atiye et al., 2005), indicating that these chromosomal regions may contain tumour suppressor genes and oncogenes involved in the development of osteosarcoma.

We have previously shown by gene expression profiling of osteosarcoma cell lines that genes encoding proteins associated with the plasma membrane and the extracellular matrix were lower expressed and genes encoding nuclear proteins were higher expressed than in osteoblasts (M. Wilkens, K.A. Kooi, R.M.W. Hofstra, C.H.C.M. Buys, F. Gerbens, submitted). To investigate whether these differences in gene expression should be considered as cell line-specific or as tumour-specific, we compare in this study gene expression profiles of cultured osteoblasts with those of primary osteosarcoma tumours and the cultured osteosarcoma cell lines. Since differences in gene expression in tumours can be the result of either transcriptional regulation and/or the occurrence of chromosomal gains or losses, we also determined copy number changes in primary osteosarcoma by array-CGH.

Material & Methods

Patients and cell lines

One female and five male patients with osteosarcoma were included in this study. Moreover four female and one male patient undergoing total knee replacement surgery were included as donors of trabecular bone fragments. All samples were collated and used in accordance with the ethical regulations of the hospital.
Five human osteoblastic osteocarcoma cell lines namely MG63 (ATCC CRL-1427), SaOS-2 (ATCC-HTB-85), U-2OS (ATCC CRL-1543), HOS (ATCC-HTB-96) and G292 (ATCC-CRL-1423) were obtained from the American Type Culture Collection (ATCC, Baltimore, MD USA).

Tumour tissue collection and processing

Osteosarcoma samples were collected from freshly frozen surgically resected specimens obtained before chemotherapy. Moreover a biopsy from one of the tumours taken 5 weeks before tumour resection was included. The pathologist classified three of these tumours as osteoblastic osteosarcoma and three as fibroblastic osteosarcoma. Tissue sections containing at least 80% tumour cells as revealed by haematoxylin and eosin staining were used for total RNA and DNA isolation.

Culturing of primary osteoblasts and osteosarcoma cell lines

Trabecular bone fragments were treated as described by Scheven et al. (1991) to obtain primary osteoblastic cells. Specimens were transported under sterile conditions from the operating centre to the laboratory, chopped into pieces of 0.2 cm – 1.0 cm in diameter and washed three times with sterile phosphate-buffered saline. Bone fragments were treated with collagenase (1 mg/ml) (GIBCO - Invitrogen Inc, Carlsbad, Ca, USA) at 37°C during 2.5 h and again washed three times with sterile phosphate-buffered saline. Subsequently, the bone fragments were cultured in Dulbecco‘s modified Eagle medium (DMEM) with l-glutamax (GIBCO - Invitrogen) supplemented with 100 IU/ml penicillin and 1000 µg/ml streptomycin (all from GIBCO - Invitrogen) and 10% foetal bovine serum (Biowhittaker – Cambrex, Verviers,

Belgium) in small culture flasks (25 cm^2) at 37°C in a humidified atmosphere with 5% CO_2. Cells isolated as osteoblasts were harvested for RNA isolation after reaching 70% - 80% of confluence by trypsin (0.05%)/EDTA (0.02%) (GIBCO – Invitrogen) treatment, pelleted, washed with sterile phosphate-buffered saline, pelleted again and stored at –80°C until RNA isolation. Characterisation of the isolated osteoblasts was performed by comparison to fibroblasts through real-time RT-PCR analysis of six tissue specific genes (Marga Wilkens, Krista A. Kooi, Edo A.D. Plantinga, Gerard J. te Meerman, Robert M.W. Hofstra, Charles H.C.M. Buys and Frans Gerbens, submitted).

The human osteoblastic osteosarcoma cell lines MG63, SaOS-2 and HOS were cultured on RPMI1640 (GIBCO – Invitrogen), while U-2 OS and G292 were grown on McCoy`s 5A (GIBCO - Invitrogen). Both media were supplemented with 10% foetal bovine serum (Biowhittaker), 2 mM glutamine, 100 U/ml penicillin, 1000 μg/ml streptomycin and 250 μg/ml fungizone amphotericine B (all from GIBCO - Invitrogen). All cell lines were cultured in culture flasks at 37°C in a humidified atmosphere with 5% CO_2. After having reached 70% to 80% of confluence, human osteosarcoma cells were harvested by trypsin (0.05%)/EDTA (0.02%) (GIBCO - Invitrogen) treatment, pelleted, washed with sterile phosphate-buffered saline, pelleted again and stored at –80°C until RNA isolation.

RNA isolation

Total RNA was isolated with the RNeasy mini kit including DNase treatment (Qiagen, Valencia, CA, USA). Total RNA yield and purity was assessed by measuring absorbances at 260 nm and 280 nm spectrophotometrically on a NanoDrop® ND-1000 UV-VIS spectrophotometer (Nanodrop technologies, Wilmington, DE, USA). RNA integrity was determined by 1% formaldehyde agarose gel electrophoresis.

DNA isolation

Genomic DNA was isolated as described by Van den Berg (2003) and subsequently treated with RNase A (Roche, Indianapolis, IN, USA) as described by the manufacturer. Genomic DNA will be purified with YM-30 columns (Millipore Corp, Bedford, MA, USA) after proteinase K (Sigma-Aldrich, St. Louis, MO, USA)

treatment. Total DNA yield and purity were calculated by measuring absorbances at 260 nm and at 280 nm spectrophotometrically on a NanoDrop® ND-1000 UV-VIS spectrophotometer (Nanodrop technologies).

Transcriptional profiling

For transcriptional profiling 2 µg of total RNA was used for linear amplification of mRNA transcripts according to the Dutch Cancer Institute protocol (www.nki.nl/nkidep/pa/microarray/protocols.htm). The resulting purified double stranded cDNA was transcribed *in vitro* with the T7 Megascript kit (Ambion, Huntingdon, UK) as described by the manufacturer with a final concentration of 7.5 mM for all NTPs and replacing UTP with aminoallyl-UTP/UTP (1:1) ('t Hoen et al., 2003). Amplified RNA (aRNA) was purified with the RNeasy kit (Qiagen). Subsequently, 5 µg of aRNA was labelled with monoreactive Cyanine-3 (2.5 nmol per reaction) or Cyanine-5 (2.5 nmol per reaction) fluorophores (Amersham Biosciences, Little Chalfont, UK) to the incorporated aminoallyl-modified nucleotides. Labelled aRNA was separated from unincorporated Cyanine-3 or Cyanine-5 molecules with Microspin G-50 column chromatography (Amersham Biosciences) as described by the manufacturer.

Experimental design

A randomised design was applied to identify differentially expressed genes between five-cultured primary human osteoblast, five human osteosarcoma cell lines and six primary human osteosarcoma tissue samples. Each sample was labelled with Cyanine-3 and Cyanine-5 separately and subsequently assigned at random to a sample of the opposite colour.

Micro-array Slides & Hybridisation

Transcriptional profiling experiments were performed using in-house manufactured micro-arrays containing 21,329 gene specific 70-mer oligonucleotides from the human oligonucleotide library version 2.1 (Operon Biotechnologies Inc., Huntsville, AL, USA) printed at 10 pM on UltraGAPS coated slides (Corning Life Sciences BV, Schiphol-Rijk, The Netherlands). Slide blocking, prehybridisation and hybridisation

were performed as described by Hegde et al (2000) with some modifications. In short, slides were blocked with ethanolamine at 52°C during 1 h. Prehybridisation was done with prewarmed prehybridisation buffer containing 0.5% bovine serum albumin (Sigma-Aldrich) at 52°C during 45 min. Subsequently, the slides were washed 6 times with preheated water (52°C) and dried by centrifugation at 800 rpm during 3 min and immediately used for hybridisation. Labelled aRNA samples, 30 µg polyA (Sigma Aldrich) and an equal volume of preheated (52°C) 2X hybridisation buffer were mixed together, heated at 95°C for 3 min and applied to preheated micro-array slides. Hybridisation was performed at 52°C for 48 h in the dark. Subsequently, slides were washed in 5 steps each for 5 min: 1xSSC/0.2% SDS at 52ºC; 0.1xSSC/0.2% SDS at 52ºC; 0.1xSSC at 52ºC; 0.1xSSC at RT and 0.01xSSC at RT. Finally slides were dried by centrifugation at 800 rpm during 3 min and scanned with an Affymetrix GMS428^{TM} array scanner (Affymetrix Inc, Carlsbad, CA, USA).

Micro-array data analysis

Fluorescent signal intensity data for each spot and for each fluorophore were extracted from the scanned images of each micro-array slide using ImaGene 5.6 (BioDiscovery, El Segundo, CA, USA). Signal intensity ratios were log transformed and normalised by intensity dependent linear regression (loess) using the Limma package (http://bioinf.wehi.edu.au/limma) in R. Initial data reduction analysis using principal component analysis revealed no dependency between single colour readings from both fluorophores and therefore for further analysis readings were treated separately. Data analysis was performed using BRB ArrayTools v3.3 developed by Dr. Richard Simon and Amy Peng Lam (http://linus.nci.nih.gov/~brb/download.html). Basically, control spots, spots with high between-pixel-intensity variability and spots designated as bad by eye were excluded and log intensities below 9.5 were thresholded to this minimum. After excluding genes with more than 50% missing data across all observations, 19,333 genes remained. Significant differences in gene expression between the different phenotype classes, primary osteoblastic and fibroblastic osteosarcoma, osteosarcoma cell lines and cultured primary osteoblasts were identified by an univariate F-test using a significance threshold of 0.001. Moreover, multivariate permutation (Reiner et al., 2003) was applied to restrict the false discovery rate to 10% in the final set of significantly differentially expressed genes. Hierarchical clustering of the 16 duplicate samples and the 838 selected genes was based on one-minus-uncentered-clustering

using average linkage. The robustness of the clustering results was tested by applying 100 independent perturbations of the data and re-clustering (McShane et al., 2002).
Gene ontology categories with between 5 to 100 genes represented on the array that had more differentially expressed genes between the sample classes than expected by chance were identified by the permutation based Fisher (LS) and Kolmogorov-Smirnov (KS) statistic (nominal significance level 0.001) (Pavlidis et al., 2004).
Regions with correlated gene expression biases were determined using the cluster-along-chromosomes procedure as implemented in CGH-Miner and restricting the false discovery rate to 1% compared to well matched self-self hybridisations (Wang et al., 2005). The signal intensities from transcriptional profiling of each primary osteoblastic or fibroblastic osteosarcoma tumour sample relative to the mean signal intensity of all primary osteoblast cultures was determined, smoothed with a moving average window of 5 genes and clustered along individual chromosome arms.

Comparative Genomic Hybridisation Arrays

The genome-wide micro-array used in this study consists of 6,465 large-insert clones, including both bacterial artificial chromosomes (BACs) and P1-based artificial chromosomes (PACs). Clones were selected from the 1-Mb BAC collection obtained from Dr. Nigel Carter (Wellcome Trust Sanger Institute, Cambridge, UK), supplemented with clones from the Human BAC Resource Consortium-1 Set (Dr. Pieter de Jong, Children's Hospital Oakland Research Institute, USA). The BAC DNA isolation procedure was adapted from the protocol published at the website of Dr. Mariano Rocchi at the University of Bari (http://www.biologia.uniba.it/rmc/). For positioning the BACs with respect to the human sequence, we used the May 2004 human reference sequence (UCSC version hg17) based on NCBI Build 35. In addition, the array contained a series of control spots, including C_oT-1 DNA, total human DNA, and Drosophila DNA. DOP-PCR-amplified BAC or PAC DNA was spotted in triplicate onto epoxy-coated slides (Schott Nexterion, Mainz, Germany), using a MicroGrid II arrayer (BioRobotics, Cambridge, UK). Slides were hybridised as described (Kok et al., 2005).
Tumour DNA and matched normal genomic DNA was labelled with Cyanine-5 and Cyanine-3 respectively, using a BioPrime random labelling kit (Invitrogen, Carlsbad, CA, USA). After incubation at 37°C overnight, unincorporated nucleotides were removed by use of MicroSpin G-50 columns (Amersham Biosciences, New Jersey,

USA). Test and reference DNA were combined on a Microcon YM30 column (Millipore, Cork, Ireland) and subsequently dissolved in 80 μl (Schott Nexterion) hybridisation buffer containing 5% dextran sulphate and supplemented with 450 μg human C_oT-1 DNA and 1 mg yeast tRNA. Labelled DNA was denatured at 99°C for 10 min and kept at 65°C until hybridisation by means of a PCR machine. Hybridisations were performed under lifterslips (Erie Scientific, Portsmouth, USA) in humidified chambers at 65°C for approximately 48 h, using a shaking water bath. Post-hybridisation washes included a wash in 2*SSC/0.2% SDS at 65°C for 10 min, a wash in 2*SSC at room temperature for 5 min and a wash in 0.2*SSC at room temperature for 5 min. Slides were briefly rinsed in distilled water and immediately dried by centrifugation. Arrays were scanned using the Affymetrix 428 Scanner (Affymetrix Inc.).

CGH array data analysis

The resulting images were analysed with the Bluefuse (Bluegnome, Cambridge UK) software package and applying a “block-median” normalisation with exclusion of control spots. A BAC was eliminated if the standard deviation of a triplo representing one BAC was greater than 0.2. For each tumour, the normalised $\log_2$ ratios of all BACS were aligned according to their position on the genome to determine the copy number changes. For each primary osteoblastic or fibroblastic osteosarcoma, DNA copy number changes for each chromosomal region were identified by CGH-miner (http://www-stat.stanford.edu/~wp57/CGH-Miner) based on a moving average of the three nearest BAC`s centred on that region.

Results

Expression profiling

Using micro-arrays containing 19,333 oligonucleotide features, we could by hierarchical clustering of signal intensities discriminate between primary osteoblasts (n=5), osteosarcoma cell lines (n=5) and osteosarcoma tumour samples (n=6) with high reproducibility (R=0.998; data not shown). Within the group of six osteosarcoma tumours, fibroblastic (n=3) and osteoblastic (n=3) tumours were clearly differentiated. Moreover, to examine the reliability of the clustering procedure, the expression profile

of tumour tissue from a pre-operative biopsy was included. That showed a stronger correlation to the profile of its resected counterpart than to the other samples.
We then identified the genes that were significantly ($p < 0.001$) differentially expressed in each comparison of the three sample classes. The osteosarcoma tumours had 362 and 434 differentially expressed genes compared to the osteoblasts and the osteosarcoma cell lines, respectively. The osteosarcoma cell lines had 158 differentially expressed genes compared to the osteoblasts. In total, 838 genes co-ordinately discriminate between any individual comparison of the three sample classes (Fig. 1).

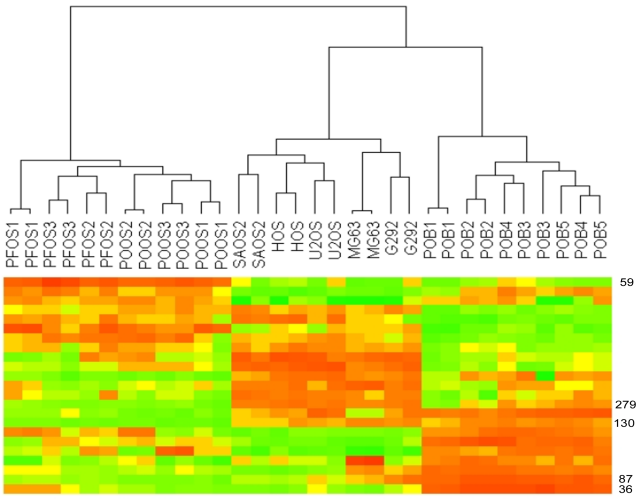


Figure 1: Heat map visualisation of two-way clustering of six osteosarcoma tumours, five osteosarcoma cell lines and five osteoblast specimens and the 838 significantly differentially expressed genes of the three comparisons between these sample classes. The clustering heat map was condensed to 23 gene clusters. POOS: primary osteoblastic osteosarcoma tumour; PFOS: primary fibroblastic osteosarcoma tumour; POB: primary osteoblast culture; SAOS2, U2OS, HOS, MG63 and G292 are osteoblastic osteosarcoma cell lines.

Tables 1, 2 and 3 show the 20 most significantly differentially expressed genes for each comparison. The complete lists of significantly differentially expressed genes indicate an over-representation of gene ontology (GO) categories for genes encoding proteins associated with lysosomes and involved in immune system related functions, such as *IgG* and *IgE* binding in the osteosarcoma tumours compared to both primary osteoblasts and the osteosarcoma cell lines. These genes have elevated levels of expression in the osteosarcoma tumours. Genes encoding proteins associated with cell adhesion and the extracellular matrix, including matrix

metalloproteinases and collagens, have lower expression in osteosarcoma tumours and osteosarcoma cell lines than in osteoblasts. Genes belonging to GO categories associated with cell cycle regulation, such as DNA replication and DNA repair, had an elevated expression in the osteosarcoma cell lines compared to the primary osteoblasts. Although these latter GO categories were not identified between primary osteoblasts and the osteosarcoma tumours, the comparison of the osteosarcoma tumours with the osteosarcoma cell lines revealed that similar cell cycle regulation-associated categories were elevated in the osteosarcoma cell lines.

Table 1: Twenty most differentially expressed significant genes in six osteosarcoma tumours compared to cultures of five primary osteoblast specimens.

GenBank Accession	*Gene Symbol*	*Gene coding for*	*Fold Difference*
Higher expressed			
AL121939	*PRSS35*	Protease, serine, 35	14.7
J04162	*FCGR3A*	Fc fragment of IgG, low affinity IIIa, receptor (CD16a)	14.3
NM_031311	*CPVL*	Carboxypeptidase, vitellogenic-like	12.7
NM_004684	*SPARCL1*	SPARC-like 1 (mast9, hevin)	11.8
NM_003890	*FCGBP*	Fc fragment of IgG binding protein	10.8
NM_003332	*TYROBP*	TYRO protein tyrosine kinase binding protein	9.4
NM_012072	*C1QR1*	Complement component 1, q subcomponent, receptor 1	8.9
NM_003177	*SYK*	Spleen tyrosine kinase	8.8
NM_001175	*ARHGDIB*	Rho GDP dissociation inhibitor (GDI) beta	8.3
NM_000014	*A2M*	Alpha-2-macroglobulin	7.9
NM_006762	*LAPTM5*	Lysosomal associated multispanning membrane protein 5	7.4
NM_015991	*C1QA*	Complement component 1, q subcomponent, alpha polypeptide	7.4
NM_004877	*GMFG*	Glia maturation factor, gamma	6.9
NM_021642	*FCGR2A*	Fc fragment of IgG, low affinity IIa, receptor (CD32)	6.2
NM_013322	*SNX10*	Sorting nexin 10	5.8
NM_031310	*PLVAP*	Plasmalemma vesicle associated protein	5.7
AF212221	*MYLIP*	Myosin regulatory light chain interacting protein	5.7
NM_005211	*CSF1R*	Colony stimulating factor 1 receptor, formerly McDonough feline sarcoma viral (v-fms) oncogene homolog	5.5
NM_004106	*FCER1G*	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	5.1
NM_004001	*FCGR2B*	Fc fragment of IgG, low affinity IIc, receptor for (CD32)	5.1
Lower expressed			
NM_002421	*MMP1*	Matrix metalloproteinase 1 (interstitial collagenase)	29.1
NM_002422	*MMP3*	Matrix metalloproteinase 3 (stromelysin 1, progelatinase)	27.6
NM_003881	*WISP2*	WNT1 inducible signaling pathway protein 2	23.5
AB051431	*KIAA1644*	KIAA1644 protein	20.4
NM_001552	*IGFBP4*	Insulin-like growth factor binding protein 4	17.8
NM_002852	*PTX3*	Pentraxin-related gene, rapidly induced by IL-1 beta	15.1
NM_004598	*SPOCK*	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican)	14.7
NM_000600	*IL6*	Interleukin 6 (interferon, beta 2)	12.9
NM_032849	*FLJ14834*	Hypothetical protein FLJ14834	12.4
BC015134	*THBS1*	Thrombospondin 1	12.3
NM_052947	*ALPK2*	Alpha-kinase 2	11.7
NM_031957	*KRTAP1-5*	Keratin associated protein 1-5	11.6
NM_000820	*GAS6*	Growth arrest-specific 6	11.5
NM_006851	*GLIPR1*	GLI pathogenesis-related 1 (glioma)	11.2
NM_021570	*BARX1*	BarH-like homeobox 1	10.8
NM_015429	*ABI3BP*	ABI gene family, member 3 (NESH) binding protein	10.1
AF109161	*CITED2*	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	9.9
NM_000428	*LTBP2*	Latent transforming growth factor beta binding protein 2	9.8
NM_000956	*PTGER2*	Prostaglandin E receptor 2 (subtype EP2), 53kDa	9.6
NM_001850	*COL8A1*	Collagen, type VIII, alpha 1	9.4

Table 2: Twenty most differentially expressed significant genes in six osteosarcoma tumours compared to cultures of five osteosarcoma cell lines.

GenBank Accession	*Gene Symbol*	*Gene coding for*	*Fold Difference*
Higher expressed			
NM_014713	*LAPTM4A*	Lysosomal-associated protein transmembrane 4 alpha	17.2
NM_004450	*ERH*	Enhancer of rudimentary homolog (Drosophila)	16.9
NM_000900	*MGP*	Matrix Gla protein	15.6
NM_002085	*GPX4*	Glutathione peroxidase 4 (phospholipid hydroperoxidase)	14.7
NM_003332	*TYROBP*	TYRO protein tyrosine kinase binding protein	13.0
J04162	*FCGR3A*	Fc fragment of IgG, low affinity IIIa, receptor (CD16a)	13.0
D86978	*NUP205*	Nucleoporin 205kDa	12.0
NM_005410	*SEPP1*	Selenoprotein P, plasma, 1	10.1
NM_006762	*LAPTM5*	Lysosomal associated multispanning membrane protein 5	9.9
NM_000302	*PLOD1*	Procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1	9.4
NM_022763	*FNDC3B*	Fibronectin type III domain containing 3B	9.3
NM_012072	*C1QR1*	Complement component 1, q subcomponent, receptor 1	8.9
NM_004684	*SPARCL1*	SPARC-like 1 (mast9, hevin)	8.8
NM_005211	*CSF1R*	Colony stimulating factor 1 receptor, formerly McDonough feline sarcoma viral (v-fms) oncogene homolog	8.4
NM_004877	*GMFG*	Glia maturation factor, gamma	8.2
NM_001129	*AEBP1*	AE binding protein 1	7.7
NM_016650	*MS4A4A*	Membrane-spanning 4-domains, subfamily A, member 4	7.3
NM_000591	*CD14*	CD14 antigen	6.6
AY043362	*KIF1B*	Kinesin family member 1B	6.1
NM_004106	*FCER1G*	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	6.0
Lower expressed			
NM_006115	*PRAME*	Preferentially expressed antigen in melanoma	14.3
NM_004060	*CCNG1*	Cyclin G1	10.3
NM_002061	*GCLM*	Glutamate-cysteine ligase, modifier subunit	9.5
NM_016441	*CRIM1*	Cysteine rich transmembrane BMP regulator 1 (chordin-like)	8.2
NM_001151	*SLC25A4*	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 6	8.0
NM_001730	*KLF5*	Kruppel-like factor 5 (intestinal)	7.5
NM_000071	*CBS*	Cystathionine-beta-synthase	7.3
NM_032181	*FLJ13391*	Hypothetical protein FLJ13391	7.1
AK024263	*SLC38A1*	Solute carrier family 38, member 1	6.9
NM_001458	*FLNC*	Filamin C, gamma (actin binding protein 280)	6.4
NM_003201	*TFAM*	Transcription factor A, mitochondrial	6.4
NM_003104	*SORD*	Sorbitol dehydrogenase	6.4
NM_003100	*SNX2*	Sorting nexin 2	6.1
AF104032	*SLC7A5*	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	6.1
NM_022909	*CENPH*	Centromere protein H	6.0
AF109161	*CITED2*	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	5.8
NM_003078	*SMARCD3*	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 3	5.7
NM_001709	*BDNF*	Brain-derived neurotrophic factor opposite strand	5.7
AK055539	*LOC143903*	Layilin	5.5
NM_003000	*SDHB*	Succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	5.5

Table 3: Twenty most differentially expressed significant genes in five osteosarcoma cell lines compared to cultures of five primary osteoblast specimens.

GenBank Accession	*Gene Symbol*	*Gene coding for*	*Fold Difference*
Higher expressed			
NM_006115	*PRAME*	Preferentially expressed antigen in melanoma	18.5
NM_000946	*PRIM1*	Primase, polypeptide 1, 49kDa	6.3
NM_024037	*MGC2603*	Chromosome 1 open reading frame 135	5.8
NM_032117	*GAJ*	GAJ protein	5.7
NM_016623	*FAM49B*	Family with sequence similarity 49, member B	5.6
NM_024094	*DCC1*	Defective in sister chromatid cohesion homolog 1 (S. cerevisiae)	5.6
NM_033380	*COL4A5*	Collagen, type IV, alpha 5 (Alport syndrome)	5.3
BC016950	*MGC2610*	Kelch-like 23 (Drosophila)	5.2
NM_012198	*GCA*	Grancalcin, EF-hand calcium binding protein	5.1
NM_014791	*MELK*	Maternal embryonic leucine zipper kinase	4.9
AB058697	*FLJ10719*	Hypothetical protein FLJ10719	4.8
NM_004111	*FEN1*	Flap structure-specific endonuclease 1	4.8
BC010437	*MGC2610*	Kelch-like 23 (Drosophila)	4.3
BC011000	*CDCA5*	Cell division cycle associated 5	4.2
NM_002646	*PIK3C2B*	Phosphoinositide-3-kinase, class 2, beta polypeptide	4.1
NM_021238	*C12orf14*	Family with sequence similarity 60, member A	4.0
NM_014109	*ATAD2*	ATPase family, AAA domain containing 2	4.0
BC009435	*C9orf140*	Chromosome 9 open reading frame 140	4.0
NM_004153	*ORC1L*	Origin recognition complex, subunit 1-like (yeast)	3.9
BC013136	*MTBP*	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse) binding protein, 104kDa	3.9
Lower expressed			
NM_015429	*ABI3BP*	ABI gene family, member 3 (NESH) binding protein	43.5
NM_001078	*VCAM1*	Vascular cell adhesion molecule 1	35.7
NM_003881	*WISP2*	WNT1 inducible signaling pathway protein 2	33.3
NM_001353	*AKR1C1*	Aldo-keto reductase family 1, member C1	25.0
NM_005807	*PRG4*	Proteoglycan 4	14.9
AF109681	*ITGA11*	Integrin, alpha 11	14.5
NM_002427	*MMP13*	Matrix metalloproteinase 13 (collagenase 3)	14.3
NM_002402	*MEST*	Mesoderm specific transcript homolog (mouse)	13.7
BC016964	*MRGPRF*	MAS-related GPR, member F	11.6
NM_001850	*COL8A1*	Collagen, type VIII, alpha 1	11.6
NM_000095	*COMP*	Cartilage oligomeric matrix protein	9.8
NM_032035	*LTBP2*	Latent transforming growth factor beta binding protein 2	9.6
NM_020404	*CD164L1*	CD248 antigen, endosialin	9.4
M35878	*IGFBP3*	Insulin-like growth factor binding protein 3	9.3
AK021531	*COL3A1*	Collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	9.2
NM_001964	*EGR1*	Early growth response 1	9.1
NM_000396	*CTSK*	Cathepsin K (pycnodysostosis)	8.8
NM_000963	*PTGS2*	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	8.7
NM_007361	*NID2*	Nidogen 2 (osteonidogen)	8.6
NM_022842	*CDCP1*	CUB domain containing protein 1	8.5

Functional analysis

We identified also GO categories that were significantly different between each of the osteoblast, osteosarcoma cell line and osteosarcoma tumour classes (Table 4). In the comparison between osteosarcoma tumours and osteoblasts, many of significantly differentially expressed genes belonged to the GO categories related to

immunoglobulin binding and angiogenesis. On the other hand, many genes from GO categories related to transcriptional and translational machinery occurred in the list of genes significantly differentially expressed between osteosarcoma tumours and the osteosarcoma cell lines. The main functional differences between osteosarcoma cell lines and the primary osteoblasts were elevated cell cycling and DNA replication activity and diminished extracellular matrix constitution.

Table 4: Gene ontology categories that are significantly ($p < 0.001$) differentially regulated for each of the three comparisons between cultures of five primary osteoblast specimens, five osteosarcoma cell lines and six osteosarcoma tumour samples.

Rank	*GO ID*	*GO description*	*N (genes)*
Osteosarcoma Tumours versus Osteoblasts			
1	0008484	Sulfuric ester hydrolase activity	15
2	0019865	Immunoglobulin binding	11
3	0006817	Phosphate transport	85
4	0001568	Blood vessel development	51
5	0001944	Vasculature development	51
6	0048514	Blood vessel morphogenesis	51
7	0004622	Lysophospholipase activity	8
8	0001525	Angiogenesis	50
9	0005520	Insulin-like growth factor binding	20
10	0019838	Growth factor binding	50
11	0030574	Collagen catabolism	15
12	0045619	Regulation of lymphocyte differentiation	9
13	0001775	Cell activation	90
14	0008238	Exopeptidase activity	89
Osteosarcoma Tumours versus Osteosarcoma Cell Lines			
1	0005666	DNA-directed RNA polymerase III complex	8
2	0005667	Transcription factor complex	94
3	0005681	Spliceosome complex	50
4	0005730	Nucleolus	91
5	0005743	Mitochondrial inner membrane	97
6	0005759	Mitochondrial matrix	60
7	0005819	Spindle	66
8	0016591	DNA-directed RNA polymerase II, holoenzyme	65
9	0003743	Translation initiation factor activity	70
10	0004812	tRNA ligase activity	50
11	0004843	Ubiquitin-specific protease activity	61
12	0008452	RNA ligase activity	50
13	0016875	Ligase activity, forming carbon-oxygen bonds	50
14	0016876	Ligase activity, forming aminoacyl-tRNA and related compounds	50
Osteosarcoma Cell Lines versus Osteoblasts			
1	0000775	Chromosome, pericentric region	30
2	0005581	Collagen	37
3	0005583	Fibrillar collagen	11
4	0005635	Nuclear membrane	87
5	0005643	Nuclear pore	45
6	0005657	Replication fork	17
7	0005819	Spindle	66
8	0046930	Pore complex	45
9	0005201	Extracellular matrix structural constituent	88
10	0000075	Cell cycle checkpoint	40
11	0000079	Regulation of cyclin dependent protein kinase activity	37
12	0000082	G1/S transition of mitotic cell cycle	54
13	0000086	G2/M transition of mitotic cell cylce	48
14	0006261	DNA-dependent DNA replication	65

Osteosarcoma tumour types

We found 92 genes to be significantly ($p<0.001$) differentially expressed between osteoblastic and fibroblastic osteosarcoma tumours. The 10 most significantly

differentially expressed genes are shown in Table 5. For several genes the measured fold differences might be underestimated because no signal intensity could be detected for either phenotype group. Functional analysis revealed the following GO categories to be significant: protein heterodimerisation activity, DNA-dependent DNA replication, endoplasic reticulum to Golgi transport, nuclear pore, pore complex, replisome, replication fork, RNA splicing via trans-esterification reactions, nuclear mRNA splicing via spliceosome, mismatch repair, maintenance of fidelity during DNA-dependent DNA replication, nucleotide-excision repair. Genes in each of these GO categories were generally higher expressed in the osteoblastic osteosarcoma samples except for the nuclear pore and pore complex categories, which were more heterogeneous.

Table 5: Ten most significantly differentially expressed genes in three osteoblastic osteosarcoma tumours compared to three fibroblastic osteosarcoma tumours.

GenBank Accession	*Gene coding for*	*Gene Symbol*	*Fold Difference*
Higher expressed			
NM_005181	Carbonic anhydrase III, muscle specific	*CA3*	38.5
AB033015	KIAA1189	*KIAA1189*	10.8
NM_032249	hypothetical protein DKFZp434F1819	*DKFZp434F1819*	6.8
NM_021939	FK506 binding protein 10, 65 kDa	*FKBP10*	4.9
NM_002014	FK506 binding protein 4, 59kDa	*FKBP4*	4.7
NM_004069	Adaptor-related protein complex 2, sigma 1 subunit	*AP2S1*	4.3
AK056243	GLI-Kruppel family member HKR1	*HKR1*	3.5
NM_018040	G patch domain containing 2	*GPATC2*	3.5
AF241785	KIAA1128	*KIAA1128*	3.1
NM_004706	Rho guanine nucleotide exchange factor (GEF) 1	*ARHGEF1*	2.9
Lower expressed			
NM_005545	Immunoglobulin superfamily containing leucine-rich repeat	*ISLR*	35.0
AF052115	A disintegrin and metalloproteinase domain 23	*ADAM23*	10.6
AF178532	Beta-site APP-cleaving enzyme 2	*BACE2*	7.4
NM_003890	Fc fragment of IgG binding protein	*FCGBP*	6.5
AK055710	Early B-cell factor 3	*EBF3*	6.2
BC004930	Zinc finger protein 614	*ZNF614*	5.2
NM_000362	Tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)	*TIMP3*	4.7
NM_032333	Chromosome 10 open reading frame 58	*C10orf58*	4.5
AK056154	Hypothetical protein FLJ12681	*FLJ12681*	3.7
AK022970	Zinc finger, CSL-type containing 2	*ZCSL2*	2.8

The selected genes are listed according to fold difference

Regions with correlated gene expression biases

We identified 11 regions that had correlated changes for all six osteosarcoma samples (data not shown). Six of these regions contained clusters of closely related genes. Four of these gene clusters showed co-ordinately elevated expression and all

were associated with immunity-related functions. These clusters included complement components (1p36; genes *C1QA, C1QG* and *C1QB*), immunoglobulin binding (1q23; genes *FCGR2A, FCGR3A, FCGR2B* and *SDHC*), immune-associated nucleotide binding proteins (7q36.1; genes *GIMAP2, GIMAP4, GIMAP5, GIMAP6*, *LR8* and *HCA112*) and macrophage dependent inflammatory proteins (17q12; genes *CCL3, CCL4* and *CCL18*). The remaining two clusters were the matrix metalloproteinase cluster (*MMP1, MMP8, MMP10, MMP12* and *MMP13*) at 11q22.2-q22.3 and the keratin associated protein cluster at 17q12-q21 (from *KRTAP3-3* to *KRTHA2*), that both had diminished expression in all osteosarcoma tissue samples. Other regions with significant gene expression biases were detected on 6q21 (*FYN*, *WISP3, TUBE1, TRAF3IP2* and *REV3L*) with diminished expression and 12p12-p13 (*ARHGDIB, RERG* and *PTPRO*) with elevated expression in the osteosarcoma samples.

Genetic aberrations determined in primary osteoblastic and fibroblastic osteosarcoma

The three osteoblastic and three fibroblastic osteosarcoma tumours revealed multiple copy number changes. Overall, copy number losses were observed more often than copy number gains (Table 6 and Figure 2A-F). Segments of the chromosome arms 2q, 3p, 5q, 6q, 9p, 10p, 11p, 13q, 16q and 20p showed loss in at least 50% of all cases. Two tumours, one osteoblastic and one fibroblastic osteosarcoma tumour, were characterised by the presence of multiple small amplicons. The most striking amplification was seen at 12q in the fibroblastic osteosarcoma tumour (Fig. 2F). In this small series, a loss of 1p, 10q, 11p and 12q appeared to be limited to osteoblastic osteosarcoma (Table 6), whereas losses at 2p, 8p and 15q appeared to be limited to fibroblastic osteosarcoma. In this small series, the most striking difference between the osteoblastic osteosarcoma and the fibroblastic osteosarcoma tumours is seen at 12q. Two out of three osteoblastic osteosarcoma tumours showed loss for this chromosome arm, whereas no loss was seen in the fibroblastic osteosarcoma. In contrast, two out of three fibroblastic osteosarcoma tumours showed a gain, or even amplification, of a segment of this chromosome arm.

Table 6: DNA copy number changes in three osteoblastic and three fibroblastic osteosarcomas.

	Osteoblastic Osteosarcoma				*Fibroblastic Osteosarcoma*			
Chromosome	Gain	Loss	Frequency gain	Frequency loss	Gain	Loss	Frequency gain	Frequency loss
1p	+	-	1/3	2/3				
1q		-		1/3		-		1/3
2p						-		2/3
2q		-		1/3		-		2/3
3p		-		2/3		-		1/3
3q								
4p		-		1/3		-		1/3
4q								
5p		-		1/3		-		1/3
5q		-		1/3		-		2/3
6p								
6q		-		2/3		-		2/3
7p	A	-	1/3	1/3				
7q	+	-	1/3	1/3				
8p						-		2/3
8q	A		1/3		A		1/3	
9p	A	-	1/3	1/3	+	-	1/3	2/3
9q		-		1/3		-		1/3
10p		-		2/3		-		1/3
10q		-		2/3				
11p		-		3/3				
11q		-		1/3		-		1/3
12p	A		1/3			-		1/3
12q		-		2/3	A		2/3	
13		-		3/3		-		2/3
14	+		1/3			-		1/3
15						-		2/3
16p		-		1/3		-		1/3
16q		-		1/3	A	-	1/3	2/3
17p		-		1/3				
17q	+	-	1/3	1/3	+	-	1/3	1/3
18p		-		1/3				
18q		-		1/3		-		1/3
19p	+		1/3		+		1/3	
19q					+		1/3	
20p		-		2/3		-		2/3
20q					+		2/3	
21	+	-	1/3	1/3		-		1/3
22		-		1/3				

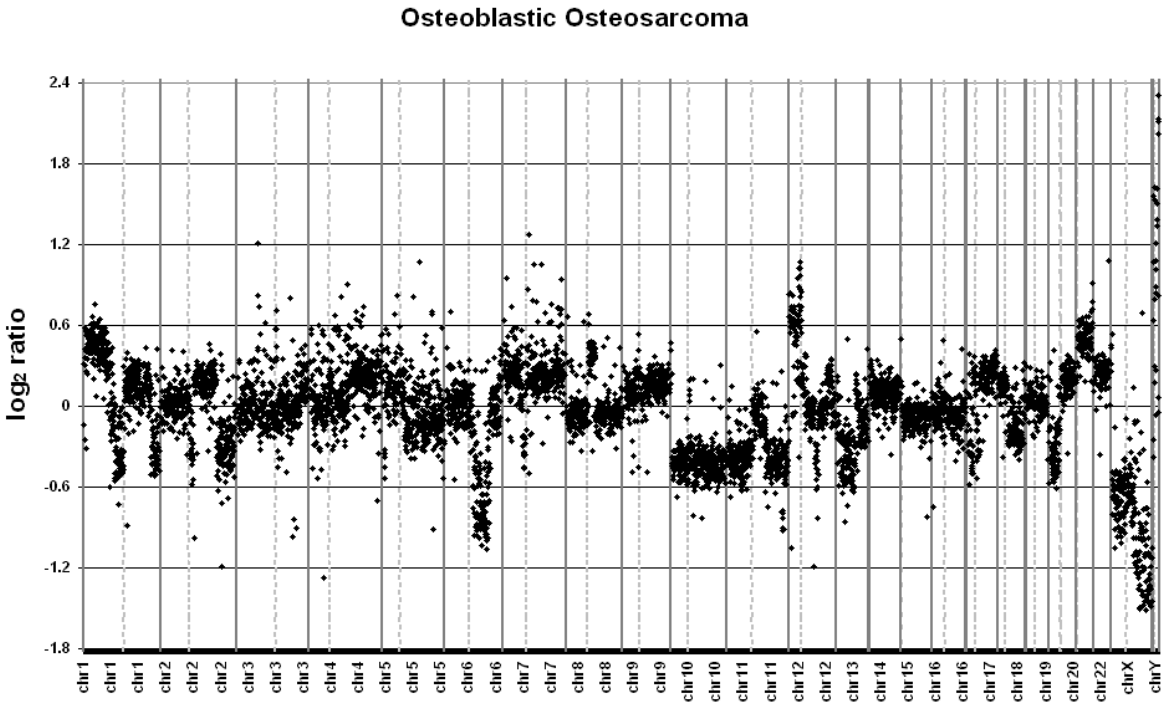
A= amplification; + = gain; - = loss

Light grey indicates a loss, gain or amplification in both osteoblastic and fibroblastic osteosarcoma

Dark grey indicates a loss, gain or amplification in osteoblastic or fibroblastic osteosarcoma

Figure 2: Array-based CGH profiles showing all chromosomal abnormalities detected in this study. Figures 2A-C show the three osteoblastic osteosarcoma and Figures 2D-F show the three fibroblastic osteosarcoma.

A.



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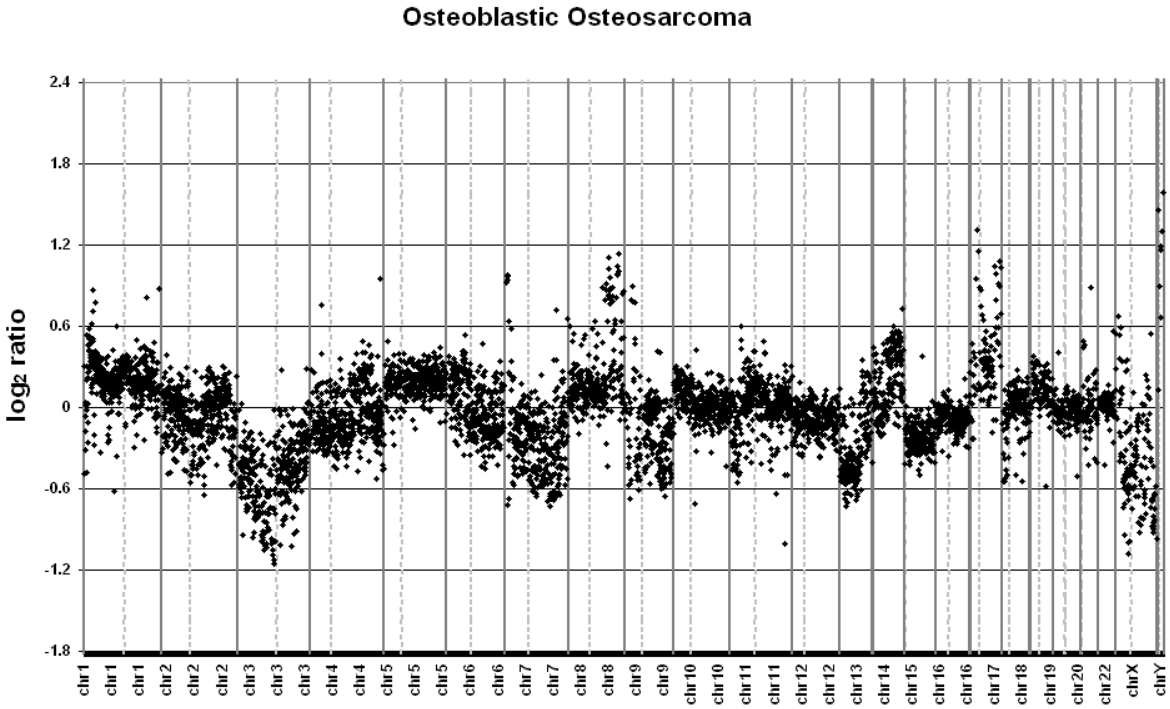
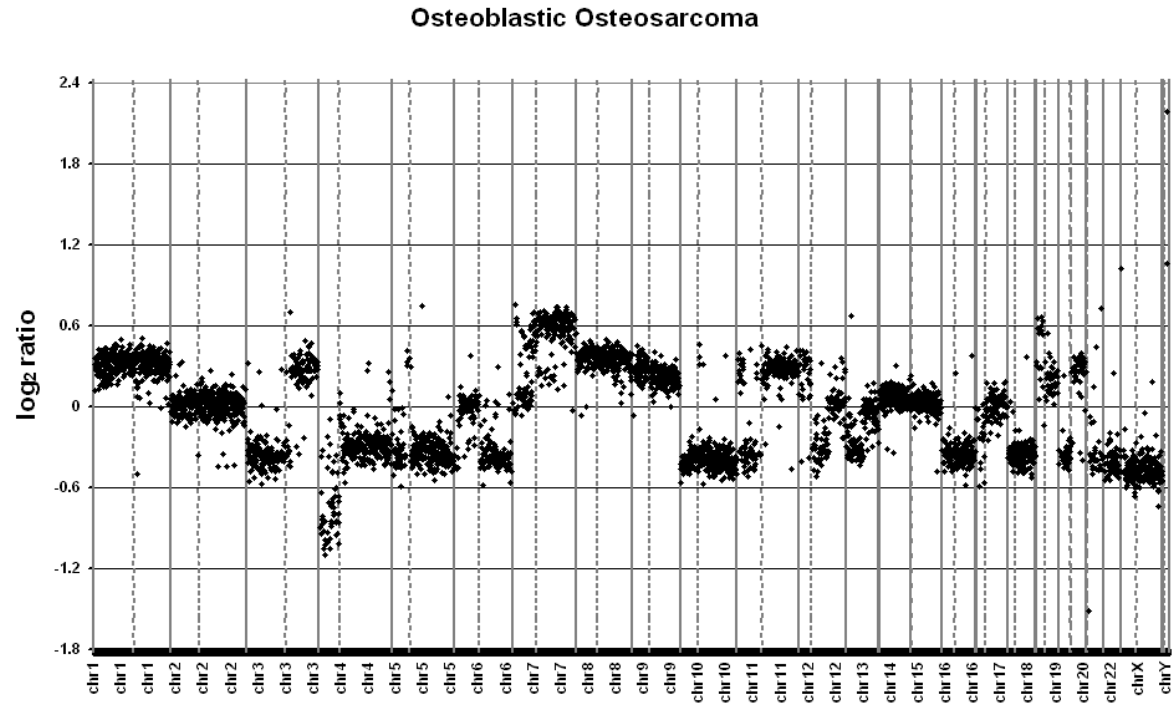
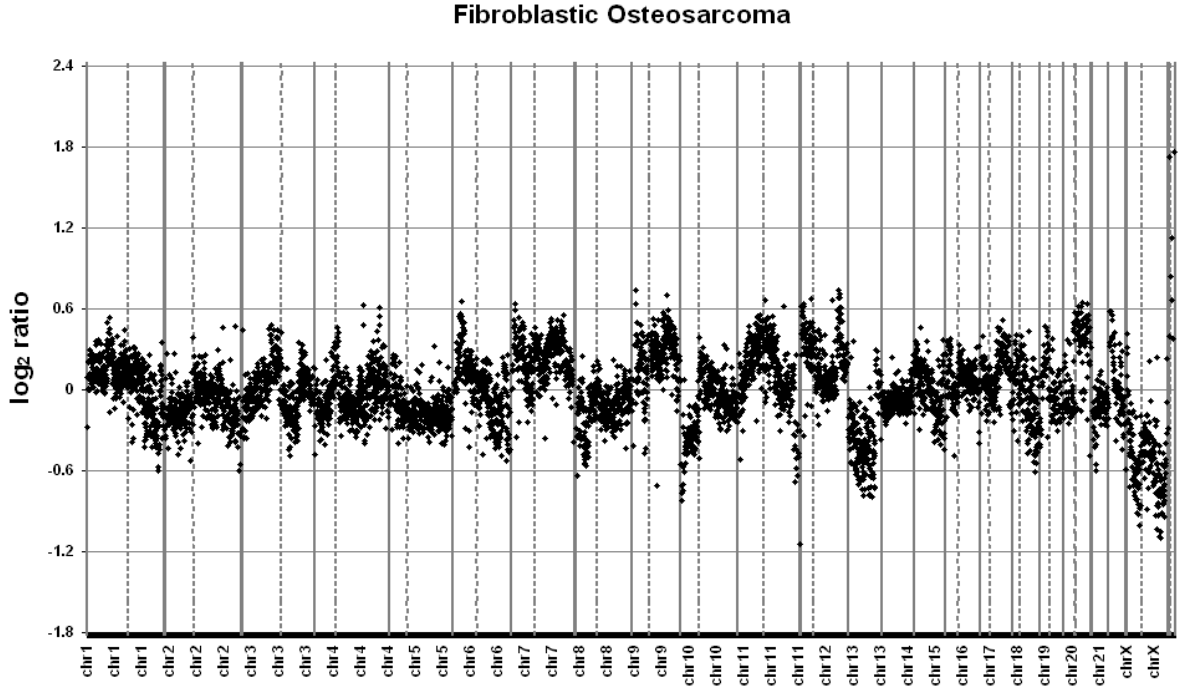


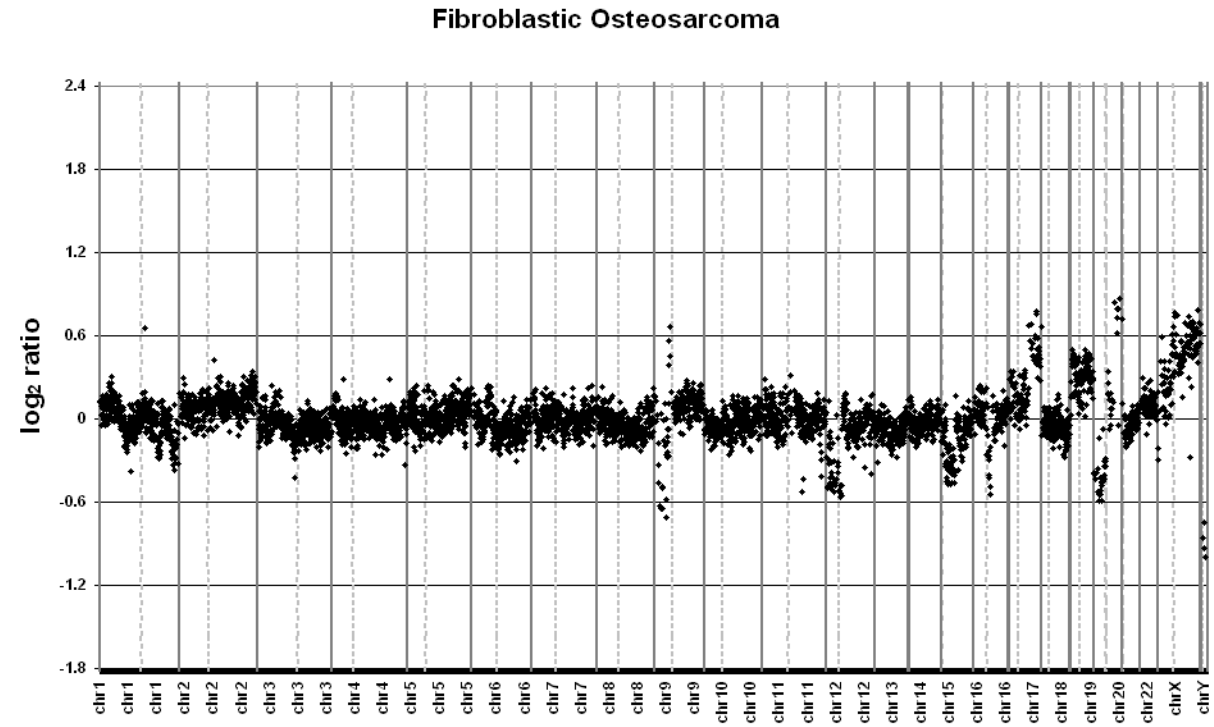
C.


Osteoblastic Osteosarcoma
log2 ratio
2.4
1.8
1.2
0.6
0
-0.6
-1.2
-1.8
chr1 chr1 chr1 chr2 chr2 chr2 chr3 chr3 chr3 chr4 chr4 chr5 chr5 chr5 chr6 chr6 chr7 chr7 chr8 chr8 chr9 chr9 chr10 chr10 chr11 chr11 chr12 chr12 chr13 chr14 chr15 chr16 chr16 chr17 chr18 chr19 chr20 chr22 chrX chrY

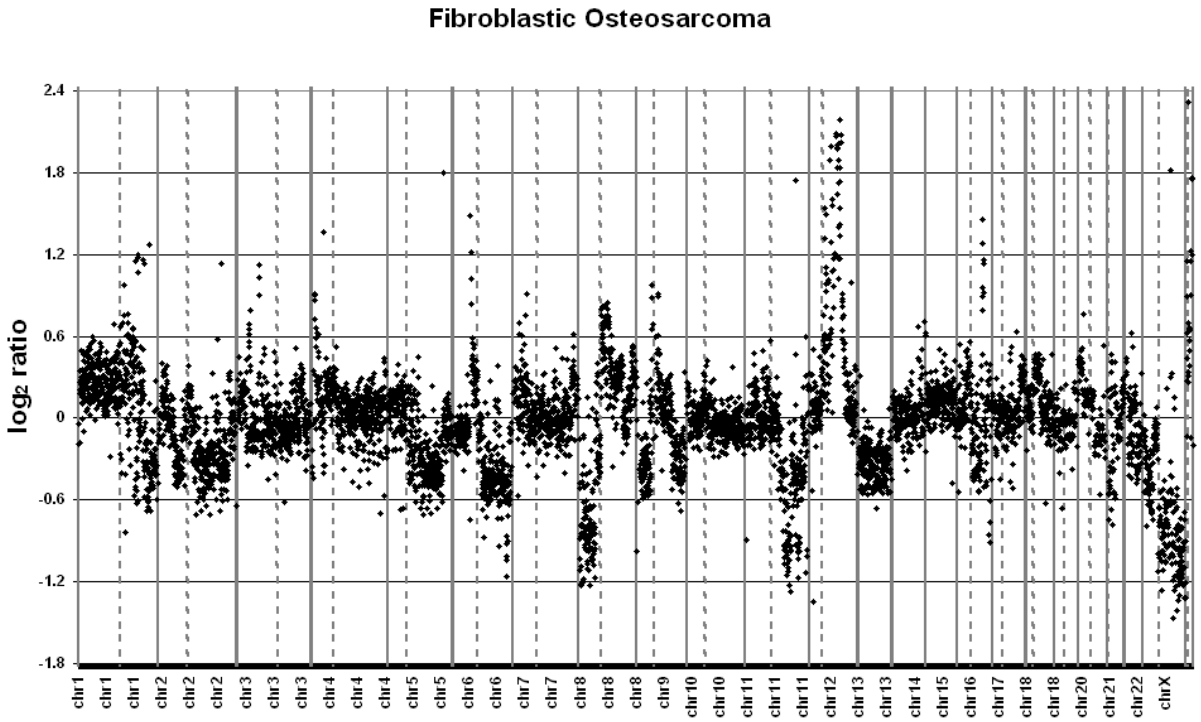
D.


Fibroblastic Osteosarcoma
log2 ratio
2.4
1.8
1.2
0.6
0
-0.6
-1.2
-1.8
chr1 chr1 chr1 chr2 chr2 chr2 chr3 chr3 chr3 chr4 chr4 chr4 chr5 chr5 chr6 chr6 chr7 chr7 chr8 chr8 chr9 chr9 chr10 chr10 chr11 chr11 chr11 chr12 chr13 chr13 chr14 chr15 chr16 chr17 chr18 chr19 chr20 chr21 chrX chrX

E.


Fibroblastic Osteosarcoma
log2 ratio
2.4
1.8
1.2
0.6
0
-0.6
-1.2
-1.8
chr1 chr1 chr1 chr2 chr2 chr2 chr3 chr3 chr3 chr4 chr4 chr5 chr5 chr5 chr6 chr6 chr7 chr7 chr8 chr8 chr9 chr9 chr10 chr10 chr11 chr11 chr12 chr12 chr13 chr14 chr15 chr16 chr16 chr17 chr18 chr19 chr20 chr22 chrX chrY

F.


Fibroblastic Osteosarcoma
log2 ratio
2.4
1.8
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0.6
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-0.6
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chr1 chr1 chr1 chr2 chr2 chr2 chr3 chr3 chr3 chr4 chr4 chr4 chr5 chr5 chr6 chr6 chr7 chr7 chr8 chr8 chr8 chr9 chr10 chr10 chr11 chr11 chr11 chr12 chr13 chr13 chr14 chr15 chr16 chr17 chr18 chr18 chr20 chr21 chr22 chrX

Discussion

In this study we compared gene expression profiles of six osteosarcoma tumour tissue samples, five osteosarcoma cell lines and five primary osteoblast cultures in order to identify genes associated with osteosarcoma development.

We identified a set of 838 genes that were significantly differentially expressed between at least two of the three-phenotype classes (Fig. 1). There are some differences in the obtained lists of significant genes between a previous and the current comparison of five osteosarcoma cell line cultures and five osteoblast cultures. These differences are the result of permitting more genes and observations in the final analysis to perform a less stringent, yet more comprehensive transcriptional analysis.

Functional analysis revealed that the most apparent differences between the osteosarcoma tumours and primary osteoblasts were genes belonging to immune system and angiogenesis associated GO categories. This might be explained by the fact that tumour tissue contains a variety of silent and activated immune cells as well as newly triggered blood vessels, whereas osteoblasts had been cultured in vitro.

Our previous results showed that the osteosarcoma cell lines had a lower expression of genes encoding membrane and extracellular matrix-associated proteins and a higher expression of genes encoding nucleus-associated proteins than primary osteoblasts, consistent with results from other tissues and cell lines (Vaes et al., 2002; Thomas et al., 2004; Eppert et al., 2005; Sandberg and Ernberg, 2005). Similarly, the osteosarcoma tumours also have a strongly repressed expression of the matrix metalloproteinase gene cluster on 11q22.2-22.3 (*MMP1, MMP3, MMP8, MMP10, MMP12* and *MMP13*), indicating reduced remodelling of the extracellular matrix compared to cultured osteoblasts. Other genes encoding extracellular matrix and membrane-associated proteins were identified either with repressed expression (*COL8A1, ITGA3, ITGA5* and *THBS1*) or with elevated expression, in the latter case mainly membrane-associated proteins from cells involved in immunological reactions. In osteosarcoma tumour tissue, genes involved in cell cycle regulation were repressed (data not shown) compared to the osteosarcoma cell lines, whereas no differences in those genes were identified when compared with primary osteoblasts. These findings likely reflect the higher proliferative activity of cell lines. Despite the well-known heterogeneous character of cell lines in general, the expression profiles of our osteosarcoma cell lines as a group are clearly different from the osteoblast cultures and the osteosarcoma tumours.

Functional analysis of osteoblastic and fibroblastic osteosarcoma tumours suggests that the former osteosarcoma type proliferates faster than the latter. Such a difference is, however, not seen in the clinic, where both tumour types are known as high-grade and have a fast proliferation.

The three osteoblastic osteosarcoma tumours and the three fibroblastic ones were screened for DNA copy number changes by array-CGH analysis. All osteoblastic and two of the fibroblastic tumours showed highly complex profiles, with large sets of datapoints at three or four different ratio levels (Figure 2A to F). This might indicate that these cell lines have a tetraploid origin. Moreover, the profiles of four of these samples showed the presence of many breakpoints that did not coincide with centromeric regions. One fibroblastic tumour appeared to be near diploid. The overall large number of chromosomal aberrations detected in our osteosarcoma tumours is in agreement with what is known from the literature.

It is difficult to give a clear-cut definition of copy number gains and losses difficult for tumours with a tetraploid origin. In stead, we have used the term over- and under-representation of genomic sequences. An over-representation is defined as more than 4 copies; under-representation is defined as having either 1 copy or 2 copies.

Under-representation of -parts of- 2q, 3p, 5q, 6q, 9p, 10p, 11p, 13q, 16q and 20p were seen in at least three out of six tumours. The 13q genomic region was under-represented most frequently, in all osteoblastic tumours and in two fibroblastic tumours. Chromosome arm 11p was under-represented in all three osteoblastic tumours. Three chromosome arms, 3q, 4q, and 6p did not show copy number changes in any of the samples. None of the chromosome arms showed a – partial – over-representation in at least 3 cases.

We have observed differences between both clinical types of osteosarcoma, osteoblastic and fibroblastic tumours. Under-representation of 1q, 10q, 11p and 12q was observed each in two of the osteoblastic osteosarcomas (11p even in all three), but not in the fibroblastic tumours. Under-representation of 2p and 8p appeared to be specific for fibroblastic cases. However, these results come from a very limited number of analysed tumours.

Two tumour samples, one fibroblastic osteosarcoma and one osteoblastic carcinoma, were characterised by multiple small regions of amplification. In the fibroblastic sarcoma, the long arm of chromosome 12 appeared to contain at least three amplicons in the segment 44.5 Mb – 93 Mb, relative to pter, 6p had two amplicons (5 Mb, ranging from 37 Mb – 42 Mb relative to pter) and chromosome 16 had one in the

region 16q22.1-q22.2 (2.3-Mb ranging from 68.2 – 70.5 Mb relative to pter), respectively.

Amplification of chromosome 12 is frequently found in human cancers (Kinzler et al., 1987; Roberts et al., 1989; Khatib et al., 1993; Ragazzini et al., 1999; Wunder et al., 1999). We found three amplicons in this region. For the first 12q13 amplicon (44.5 Mb - 48.3 Mb), gene expression changes exceeding 2-fold induction or repression were identified only for *ASB8, FKBP11* and *ARF3*. For *ASB8* and *FKBP11* gene expression was elevated and repressed, respectively, but these changes were also found for some of the other osteosarcoma tumours and most likely represent different forms of transcriptional regulation. Only *ARF3,* encoding ADP-ribosylation factor 3, was elevated more than 2-fold in this region. However, many more genes reside in this region, including *WNT1* whose activation is associated with a higher proliferation of cells and can result in oncogenesis (Polakis, 2000). The second amplicon of chromosome 12, 12q13-q14 (55.5 Mb - 58.4 Mb) showed elevated expression of *ARHGAP9, MBD6, PIP5K2C, GEFT, OS9, CDK4, MARCH-IX, TSFM, CTDSP2* and *KUB3*. Most notably, *CDK4* overexpression due to gene amplification is found in many types of sarcoma, including osteosarcoma (Khatib et al., 1993). The largest amplicon on 12q14-q21.3 (60.2 Mb - 93 Mb) contains many genes with elevated expression, of which *WIF1, LEMD3, MDM1, PTPRR* and *CRADD* are possibly the most notable. For the 6p21 amplicon (36.9 Mb - 42 Mb) several genes showed elevated or repressed expression, most pronouncedly *TBC1D22B, KIAA0082* and *CCND3*. Other genes in this region, including *MAPK14*, showed similar expression changes in other osteosarcoma tumours as well, suggesting common transcriptional regulation or effects of admixtured cells in these tumours. Chromosome 6p21 amplifications have been identified in a high frequency in osteosarcoma (Man et al., 2004) and other tumour types (Zielinski et al., 2005; Moinzadeh et al., 2005). For the 16q22.1-q22.2 region the expression of *COG4, SF3B3* and *KIAA0174* was at least two-fold increased.

The two highest amplified amplicons in the osteoblastic osteosarcoma tumour mapped at 17p (9.1 Mb, ranging from 7.6 Mb – 16.7 Mb relative to pter) and 17q (11.5 Mb, ranging from Mb position 68.2 to 17qtel), respectively. Gene expression analysis showed that many genes residing in these amplicons had at least a two-fold elevated gene expression in this tumour. Many of these genes could be denoted as potential target genes and we only mention *CDK3*, which is involved in cell cycle regulation.

Genomic regions that showed correlated gene expression biases across all osteosarcoma samples were not found by CGH-Miner analysis. Rather than being the result of copy number changes, correlated gene expression changes most likely reflect regions with common transcriptional regulation (MMP and keratin associated protein cluster) or the effect of a great admixture of inflammatory cells in the tumour samples.

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Chapter 5

Summary, General discussion and Future perspectives

Summary

Osteosarcoma is a malignant tumour of bone mostly arising in young children and adolescents. The incidence of osteosarcoma is 1.7 per million in individuals younger than 10 years of age and 8.2 per million in the age group between 10 to 19 years of age. In the Netherlands these incidences are very similar. The 5-year overall survival rate of 20% in 1970 changed to the current 55% to 70% with neoadjuvant chemotherapy and syrgery. Today the limbsalvage can be achieved in over three quarter of the patients.

Osteosarcoma can be found as part of some rare inherited (cancer) syndromes, such as hereditary retinoblastoma, Li-Fraumeni syndrome, Paget disease of bone, Rothmund-Thomsom syndrome, RAPADALINO syndrome and Werner syndrome or as sporadic osteosarcoma. Sporadic osteosarcoma is far more frequent than the inherited forms of osteosarcoma and it is this sporadic form of osteosarcoma that is the focus of this research.

It is believed that osteosarcoma is caused by multiple genetic aberrations, however, the precise combination of mutated genes is yet unknown. Several genes known to be involved in the above-mentioned inherited syndromes in which osteosarcoma can occur, have been found mutated in sporadic cases. Furthermore, in several studies sporadic primary osteosarcoma tumours and cell lines derived from osteosarcoma have been cytogenetically characterised using different techniques. Most studies found that primary osteosarcoma tumours and osteosarcoma cell lines had losses in common at 2q31.1 3p12-p14, 4p16.2, 6q12, 6q21, 7q35, 10p15.1, 10q22-q23, 11q25, 13q12.2, 13q14.3, 17p13.1, 17q21, 18q12, 18q21-q22 and 20; and gains at 1p22-p31, 1p21-q24, 1q25-q31, 4p16, 8q21, 9q24, 12q13-q15, 14q24-qter, 16p13, 17p11-p12, 19p13 and 21q22. For two chromosomal regions, 1p22 and 8q23-q24, high-level amplification was detected in both primary osteosarcoma tumours and osteosarcoma cell lines. As mentioned, multiple (aberrant) genes appear to be involved in the development of sporadic osteosarcoma. Those that are known are reviewed in Chapter 1. To obtain more insight into the molecular genetic changes underlying the development of osteosarcoma, gene expression profiling of cultured osteoblasts, osteosarcoma cell lines and primary osteosarcoma tumour tissue has been performed as well as array-CGH analysis of the osteosarcoma tumour tissue.

In Chapter 2 the isolation and molecular characterisation of osteoblasts, the precursor cell of osteosarcoma, is described. Osteoblasts are cultured out of bone fragments, but inadequate pre-processing of these bone fragments may also result in

culturing fibroblasts. Since morphologically the cultured osteoblasts cannot be discriminated from fibroblasts, we developed a test to discriminate both cell types from each other. Gene expression profiles of 10 osteoblast and 11 skin fibroblast cultures were compared using a 21,000-oligonucleotide micro-array.

This comparison resulted in many differentially expressed genes, 42 of which showed a significantly different expression between both types of cultured cells. These differentially expressed genes were specifically related to membrane proteins/processes such as cell communication, cell adhesion and receptor activity. For six of these genes expression differences defined by micro-array analysis were confirmed by an independent method, real-time RT-PCR. *VCAM1, KIAA1644, FGFR2* and *COL27A1* had a higher expression in osteoblasts, whereas *IMP-3* and *MME* were higher expressed in fibroblasts. Since for all six genes differences between all pairs of individual cultures were fully consistent, we consider expression analysis of these genes as a fast and efficient test to discriminate between cultured osteoblasts and fibroblasts.

Chapter 3 describes differences in expression profiles of 5 primary osteoblast cultures and 5 osteoblastic osteosarcoma cell lines by micro-array analysis. This analysis resulted in 286 genes significantly differentially expressed. Functional annotation of these genes revealed a lower expression in osteosarcoma cell lines of genes encoding proteins associated with the cell membrane and extracellular matrix and a higher expression of genes encoding nuclear proteins, such as cell cycle-related and DNA repair-associated proteins. Furthermore, regional expression biases in the genome of the osteosarcoma cell lines were found with an on average lower expression of the MMP gene cluster at 11q22.2 and of the KAP gene cluster at 17q21.2. An on average higher expression was found for the HOXB cluster at 17q21.3. Such co-expression of genes points to either chromosomal losses in tumours and tumour cell lines or to a common transcriptional regulation. To answer the question whether the lower expression in osteosarcoma cell lines of genes encoding membrane and extracellular matrix-associated proteins and the higher expression of genes encoding nucleus-associated proteins should be considered as cell line-specific or as tumour-specific, an additional comparison with gene expression profiles of primary osteosarcoma tumours was needed.

Therefore, in Chapter 4, array-CGH analysis and gene expression profiling of three osteoblastic osteosarcoma tumours and three fibroblastic osteosarcoma tumours were performed. Gene expression profiles of all six primary osteosarcoma tumours were compared to those of five osteosarcoma cell lines and five osteoblast cultures.

In total, 838 genes were significantly differentially expressed between any two of these groups of specimens. Functional analysis revealed that differences between primary tumour tissue on one hand and cultured osteoblasts on the other were mainly due to genes encoding proteins associated with inflammatory processes and angiogenesis. Genes encoding proteins involved in cell cycle regulation were expressed most prominently in the osteosarcoma cell lines. Regional repression of elevated expression in the genome for genes belonging to the MMP gene cluster at 11q22.2 was also identified in the osteosarcoma tumours. When genomic DNA from the six-osteosarcoma tumours was compared with normal human genomic DNA by array-CGH analysis, an under-representation of 11p was found only in the three osteoblastic tumours. This makes it more likely that this cluster of differentially expressed genes on 11q22 is due to a regional common transcriptional regulation.
By performing array-CGH we also found under-representation of 13q in five of the six tumours. Under-representation of 2p and 8p appeared to be specific for fibroblastic cases. Amplifications were detected in 13 different chromosomal regions, namely at 6p21, 6pter, 7p, 8q, 9p, 11q, 12p, 12q12, 12q13-q14, 12q14-q21.3, 16q22.1-22.2, 17p and 17q. Many of these genomic aberrations have been found before in osteosarcoma and other cancers. Gene expression analysis of these amplified regions in osteosarcoma indicate several genes with an increased expression. In these amplified regions unknown amplification target genes may be located, in addition to one already known gene, *CDK4*, which has earlier been described as amplified in osteosarcoma.

General discussion

Osteosarcoma

Osteosarcoma comprises a heterogeneous group of tumours arising from the bone and either occurs as part of a hereditary (cancer) syndrome or sporadically. Sporadic osteosarcoma, the most frequent form is the focus of this thesis, more in particular the underlying developmental mechanism for this tumour type. It is possible that genes involved in the inherited (cancer) syndromes of which osteosarcoma is part of the clinical phenotype, also play a role in the development of sporadic osteosarcoma. In addition, a number of “common cancer genes” have been found to become altered in osteosarcoma tumours. Cytogenetic studies of primary sporadic osteosarcoma show that the karyotypes of these neoplasms are very complex and make it difficult to define specific chromosome regions associated with osteosarcoma development. Although many genes and chromosomal aberrations have been identified, the developmental process of this tumour is only partly understood. In an attempt to further unravel the underlying genetic aberration involved in osteosarcoma development, we have performed both gene expression profiling and array-CGH analysis of primary osteosarcoma tumours. Since osteosarcoma develops out of osteoblasts, we have compared the gene expression pattern of osteoblasts with those of primary osteosarcoma tumours and osteosarcoma cell lines.

Osteoblast profiling

We have isolated osteoblasts out of bone fragments. Morphologically osteoblasts on culture resemble fibroblasts, another cell type that may grow out of inadequately pre-processed bone fragments, in culture. To discriminate between these cell types, we performed gene expression profiling on cultured fibroblasts and on our short term cultured osteoblasts. We observed clear differences between the cultures, allowing us to characterise osteoblasts by their gene expression pattern. In our list of genes differentially expressed between osteoblasts and fibroblasts, known osteoblast-specific markers such as *BMP2, BMP4, OCA, ALP* and *OPN* were missing. Presumably, this is partly due to the short-term culture of the cells, as osteoblasts after long-term culturing. Osteocalcin (*OCA*) and alkalic phosphatase (*ALP*) are both late stage markers of osteoblastic differentiation, expressed in mature osteoblasts

rather than in developing osteoblasts (Bennett et al., 2001). Furthermore, osteopontin (*OPN)* is excluded from our gene list of differentially expressed genes because of high individual variation, most likely due to low signal intensities. Based on our list of differentially expressed genes we propose that *VCAM1, KIAA1644, FGFR2* and *COL27A1* are "specifically" higher expressed in short-term cultured osteoblastic cells than fibroblasts and that these genes can be used in differentiating between cell types by real-time RT-PCR.

Transcriptional profiling

Transcriptional profiling is a high throughput method for defining global gene expression patterns that likely specify specific cell types (**Chapter 2**), specific tumour cell lines (**Chapter 3**) or specific primary tumours (**Chapter 4**). As we describe in **Chapters 2, 3** and **4,** we were able to find specific osteoblast, osteosarcoma cell line and primary osteosarcoma tumour profiles. Analysis of the differentially expressed genes between any individual comparisons of the three sample classes resulted in 838 genes co-ordinately discriminating between the classes as visualised in **Chapter 4, Fig. 1.** Functional analysis showed that in primary osteosarcoma gene ontology categories related to immunoglobulin binding and angiogenesis were significantly differentially expressed in comparison to primary osteoblasts. The fact that the gene ontology category immunoglobulin binding is significantly differentially expressed can be caused by admixture of the tumour tissue with normal tissue. In the osteosarcoma tumours a lower expression of the MMP matrix metalloproteinase gene cluster at 11q22.2-22.3 was observed, indicating a reduced remodelling of the extracellular matrix compared to cultured primary osteoblasts. The comparison of osteosarcoma cell lines and primary osteosarcoma tumours showed significant differences for the gene ontology categories related to transcription and the transcriptional machinery. Investigation of the functional differences between osteosarcoma cell lines and osteoblasts resulted in an elevated cell cycling and DNA replication activity and a diminished extracellular matrix constitution for the cell lines. All these genes ontology categories are represented in **Chapter 4, Table 1**. Although clear differences are observed between different sample classes, gene expression profiling results in a high degree of variation, attributable to various sources of variation in the total process of transcriptional profiling. Therefore, it is advised to standardise the experiments as much as possible or to avoid common sources of variation by using randomised experimental designs (Dobbin et al., 2005; Bammler et al., 2005).

RNA isolation

Standardisation starts with a verified method for total RNA isolation, throughout the studies described in this thesis the RNeasy method (Qiagen), also recommended by Affymetrix. Integrity of the isolated RNA is extremely important and needs to be assayed and approved before performing transcriptional profiling. We have analysed all RNA isolations by denaturing gel electrophoresis before starting the amplification and labelling procedures. The mRNA amplification protocol was applied in all our studies since short-term cultured osteoblasts as well as osteosarcoma tumour samples yield only small amounts of RNA. The efficiency of the mRNA amplifications was highly consistent across all samples used and resulted in on average 30 micrograms of amplified RNA (aRNA) starting from 2 micrograms of total RNA, resulting in a 1500-fold increase in gene specific transcripts. Labelling of the samples was performed using aminoallyl-modified UTP incorporation during amplification of mRNA and subsequent chemical coupling to Cyanine-3 and Cyanine-5 fluorophores to avoid dye incorporation biases during reverse transcription ('t Hoen et al., 2004).

The expression arrays

All transcriptional profiling experiments described in this thesis have been performed using in-house printed array slides containing 21,329 gene specific 70-mer oligonucleotides from the human oligonucleotide library version 2.1 (Operon Biotechnologies). Moreover, 4000 control spots were included to account for a-specific binding, grid positioning and spike-based normalisation. Using the January 2005 build of Entrez Gene or Locus Link about 1000 genes are represented by at least two independent oligonucleotides on the array, allowing for internal control of the gene expression data as described in **Chapter 3**.

Experimental design

Another important issue is the experimental design of micro-array experiments (Simon et al., 2002), in particular for dual colour array experiments, but also for single colour arrays/DNA chips. Several designs can be implemented, based on criteria like comparison with future experiments, experiments involving large numbers of samples, multicentre experiments, etc. In this thesis, two different design types have

been used, the reference design (**Chapter 2**) and a randomised design (**Chapters 3 and 4**). The type of experimental design was changed from reference to a randomised design when data analysis showed that the assumed competitive hybridisation on dual colour arrays was absent, resulting in two independent readings of different samples on the same array and thus allowing for randomised designs instead of direct comparisons on the same array like the reference design ('t Hoen et al., 2004). An advantage of this finding is that reference samples can be omitted and, using dye swap experiments, more observations for the same sample are generated, thereby, reducing variation of the signal intensity data. Furthermore, micro-array based signal intensity data for each sample on a dual colour array is treated as a single colour reading and analysed in that way.

Data analysis

Data analysis of micro-array based transcriptional profiling data is probably the most important issue. First, raw data has to be pre-processed before the statistical analysis can be performed. For example, spots with very low intensity signals for both samples, small area spots, spots with aberrant signal distributions, etc have to be omitted from the data. Rather than global methods of normalisation of the raw data, we applied a print-tip loess normalisation to account for technical variation like biases in micro-array fluorescence scanning and spatial effects on the slide due to printing or hybridisation. Subsequently, the normalised and filtered data was statistically analysed for biologically relevant genes using standard F-tests for pairwise significance of each gene. However, depending on the threshold for significance, the list of significant genes also includes a number of genes that are false positives. For example, pairwise tests for 10,000 genes with a significance threshold of 1% ($p<0.01$) will, by chance, result in 100 false positives. Implementing a more stringent significance threshold results in a lower number of false positive genes in the ultimate gene-list, however, the number of false negative genes, the ones that are truly different among the classes but not selected, will increase. Multivariate permutation is one of the methods to control the number of false positive genes in the gene list (Reiner et al., 2003). Here the phenotype classes to which each sample belongs are rearranged, while no class labels are lost, added or changed. In the transformed data set the significance of each gene is estimated again. This process of rearrangement of phenotype classes is performed randomly for many times and for each rearrangement of the data the number of genes significant for a given threshold

($p<0.001$) are recorded. For each significance threshold the number of significant genes in the rearranged data are determined and form a data dependent distribution. Obviously, genes significant for random rearranged data are false positives. Thus, for each significance threshold applied in the true data, the proportion of false positives can be estimated with a preset confidence, e.g. 90%.
In our analyses we have with 90% confidence no more than 10% false positives within the list of significantly differentially expressed genes. For experiments with relatively few samples the number of permutations of the data is rather limited and hence the number of false positives in the lists with significant genes may still be underestimated. Therefore, validation of our obtained micro-array data by real-time RT-PCR is essential.

Validation

In **Chapter 2** the genes that discriminate between cultured primary osteoblasts and fibroblasts based on transcriptional profiling were confirmed by real-time RT-PCR on the individual samples. Several genes that discriminated cultured primary osteoblasts from cultured osteosarcoma cell lines were confirmed as well (**Chapter 3**). Moreover, the correlation of the obtained ratios of different oligonucleotide spots representing the same gene also confirmed the discriminatory capacity of the micro-arrays and indicates that multiple independent observations per gene will improve the confidence of results. This principle is taken even further by analysing not individual genes, but gene ontology categories containing many genes at once described in **Chapter 4**.
Because of small differences in analysis settings, a comparison of the same samples can result in different lists of significantly differentially expressed genes. In **Chapter 3** the comparison of primary osteoblasts and osteosarcoma cell lines results in a top 10 list of differentially expressed genes (Table 2) whereas the same comparison in **Chapter 4** results in different top 10 lists (Table 3). Nevertheless, 7 genes of each of the top 10 lists in Table 2 of **Chapter 3** can be found in each of the top 20 lists in Table 3 of **Chapter 4.** The difference between both analyses is that the data has been pre-processed differently in **Chapter 3**. Spots with very low signal intensity were excluded, whereas in **Chapter 4** a lower threshold for low signal intensities was applied. Moreover, the threshold for excluding genes from the analysis based on the percentage of missing values was 25% in **Chapter 3** and 50% in **Chapter 4,** where in the latter case the number of samples was also larger. To retain only the most

relevant genes for each phenotype comparison, genes with more than 1 missing value within each phenotype class were afterwards manually removed from the lists of significant genes described in **Chapter 4**.

Genomic aberration in primary osteosarcoma

As described in **Chapter 4** we analysed six primary osteosarcoma tumours, including three osteoblastic tumours and three fibroblastic tumours, by whole genome array-CGH. We observed many copy number changes. Overall, copy number losses were observed more often than copy number gains. A number of the observed alterations were recurrent such as a loss of 13q, in five of the six tumours, 6q, 9q and 20p in four of the six tumours and 1q, 2q, 3p and 11p in three of the six tumours. Furthermore, the loss of 11p was only observed in osteoblastic osteosarcoma. High-level amplifications were detected for 7p and 12p in one or more osteoblastic tumours and for 12q and 16q in fibroblastic osteosarcoma. High-level amplification or gain was observed for 8q and 9p in both types of tumours.

Combining the gene expression profiling data with the aberrant chromosomal aberrations showed that the following regions were amplified in our set of primary osteosarcoma tumours 6p21, 11q22.2, 12q13-q14, 12q14-q21.3, 16q22.1-q22.2, 17p11.2-p13.2 and 17q25-qter. In these regions various genes could be identified of which the expression is most likely affected by regulation of copy numbers. In the region 12q13 several genes, including *CDK4,* were higher expressed in the osteosarcoma tumours as was already described by Wunder et al. (1999).

Future perspectives

We have identified gene expression profiles specific for cultured primary osteoblasts, osteosarcoma cell lines and primary osteosarcoma tumour tissue. Moreover, with array-CGH we provided corresponding genomic profiles of these osteosarcoma tumours. These analyses are based on restricted numbers of cultured cells or whole tumour tissue samples. From these studies two main challenges for future experiments were distilled. First, the strong admixture effect of inflammatory cells in osteosarcoma tumour tissue (chapter 4) needs to be addressed. Tumour tissue heterogeneity of the samples increases the variability of gene expression data

considerably. Isolation of true cancerous osteosarcoma cells with laser capture microdissection techniques would prevent this admixture effect and hence reduce variability of gene expression data. Secondly and equally important, the number of samples for each phenotype class need to be increased in order to more comprehensively identify genes that are involved in osteosarcoma and to better discriminate between subtypes of osteosarcoma. Challenges for micro-array experiments in general will be reduction of the variability of gene expression and to a lesser extent array-CGH data. In almost all cases a single oligonucleotide spot represents a single gene, transcript or exon on the entire array. Small variations in the fluorescent signal intensity will therefore add to the total variability across multiple arrays. Multiple spots of the same gene-specific oligonucleotide on each array would surely reduce the inherent variability of the fluorescent signal intensities within and across arrays and improve the value of the experiments.

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Chapter 6

Nederlandse samenvatting

Nederlandse samenvatting

Osteosarcoom is een kwaadaardige bot tumor die meestal voorkomt bij jonge kinderen en adolescenten. De frequentie waarin osteosarcoom wereldwijd optreedt is 1,7 per miljoen bij personen jonger dan 10 jaar en 8,2 per miljoen bij personen in de leeftijdsgroep van 10 tot 19 jaar. In Nederland zijn deze frequenties min of meer gelijk. De 5-jaars overleving na behandeling met de huidige chemotherapie varieert tussen de 55% en 70%.

Osteosarcoom kan voorkomen als onderdeel van verschillende zeldzame erfelijke (kanker) syndromen, zoals het Li-Fraumeni syndroom, Paget disease van het bot, het Rothmund-Thomsom syndroom, het RAPADALINO syndroom en het Werner syndroom. Osteosarcomen kunnen ook voorkomen als secundaire tumoren bij patiënten met erfelijk retinoblastoom. Osteosarcoom komt echter het meest frequent voor in sporadische vorm. In dit onderzoek richten we ons op het sporadisch osteosarcoom.

Osteosarcoom ontstaat in botvormende cellen, osteoblasten, door een verstoring van essentiële processen die leiden tot een ongeremde celgroei. De gedachte is, dat om een cel zodanig te veranderen dat deze zich tot een kankercel ontwikkelt er zowel grote afwijkingen in het genoom, chromosomale afwijkingen moeten optreden, alsook kleine veranderingen in genen (mutaties).

De genetische karakteristieken van het sporadische osteosarcoom en daarvan afgeleide cellijnen zijn in meerdere studies beschreven. Verschillende methoden zijn toegepast om met name chromosomale afwijkingen te karakteriseren. Daarbij wordt er onderscheid gemaakt tussen numerieke chromosoomafwijkingen (teveel of te weinig chromosomen) en structurele chromosoomafwijkingen, zoals bv. een deletie (het ontbreken van een stukje chromosoom). Al deze veranderingen dragen bij aan een ongeremde celgroei en soms ook aan de uitzaaiing van tumoren (het metastasering proces). De meeste onderzoeken aan osteosarcoom hebben verlies gevonden van materiaal van de chromosoom gebieden 2q31.1, 3p12-p14, 4p16.2, 6q12, 6q21, 7q35, 10p15.1, 10q22-q23, 11q25, 13q12.2, 13q14.3, 17p13.3, 17q21, 18q21-q22 en van chromosoom 20. Tevens is er een vermeerdering (amplificatie) van materiaal geconstateerd van de gebieden 1p22-p31, 1p21-q24, 1q25-q31, 4p16, 8q21, 9q24, 12q13-q15, 14q24-qter, 16p13, 17p11-p12, 19p13 en 21q22. Voor 1p22 en 8q23-q24 is een hoog niveau van amplificatie gevonden in zowel primaire osteosarcoom-tumoren als in osteosarcoom-cellijnen. Naast chromosomale afwijkingen zijn ook kleine veranderingen gevonden in genen. In een normale cel

worden specifieke eiwitten aangemaakt die ervoor zorgen dat cellen geen ongecontroleerde celgroei vertonen. Genen die coderen voor dergelijke eiwitten worden ook wel tumorsuppressor-genen genoemd. Mutaties in tumorsuppressor-genen veroorzaken een vermindering of zelfs volledige uitschakeling van de functie van het eiwit waar ze voor coderen. Een andere groep van genen die een belangrijke rol spelen zijn de zogenaamde oncogenen. Dit zijn groeibevorderende genen die wanneer ze door veranderingen in het chromosoom of genen geactiveerd worden, tot ongebreidelde groei kunnen leiden.

In hoofdstuk 1 wordt beschreven, wat bekend is over chromosomale afwijkingen, tumor suppressor genen en oncogenen die betrokken kunnen zijn bij de ontwikkeling van osteosarcoom.
Omdat echter nog onduidelijk is wat het precieze mechanisme van het ontstaan is en ook omdat het ontwikkelingsproces van het osteosarcoom slechts ten dele wordt begrepen, heeft dit promotie onderzoek zich ten doel gesteld meer inzicht te verkrijgen in de genetische afwijkingen die ten grondslag liggen aan de ontwikkeling van sporadische osteosarcomen. Daartoe zijn verschillen in expressie van genen bij osteoblasten (de cellen die het bot aanmaken), osteosarcoom-cellijnen en primaire osteosarcoom-tumoren bekeken met behulp van gen-expressie-profilering. Daarnaast is met behulp van de array-CGH techniek gekeken naar chromosomale afwijkingen bij osteosarcoom.

In hoofdstuk 2 wordt de isolering en karakterisering beschreven van de voorlopercel, de osteoblast. Osteoblasten worden gekweekt uit botfragmenten. Daarbij kunnen ook fibroblasten, in kweek morfologisch sterk gelijkend op osteoblasten, mee uitgroeien. Om beide celtypen van elkaar te kunnen onderscheiden zijn diverse testen beschikbaar. Die vragen echter om langdurig kweken en maken bovendien gebruik van stoffen die aanzetten tot differentiatie tot osteoblasten in een laat stadium van ontwikkeling met karakteristieke kenmerken zoals kalk depositie. Om langdurig kweken en aanzet tot differentiatie van deze cellen te vermijden, hebben we een efficiënte test ontwikkeld om beide celtypen van elkaar te kunnen onderscheiden. Met behulp van een oligonucleotide micro-array hebben we gen-expressie-profielen vergeleken van 10 osteoblast-kweken met 11 huidfibroblast-kweken. Met deze techniek kunnen we in één keer 21.000 genen bestuderen en vaststellen welke genen verschillend zijn wat betreft het expressie niveau in een celkweek, cellijn of tumor. Veel genen lieten verschillen zien in het niveau van expressie, maar 42 genen

lieten een statistisch significant verschil zien tussen beide typen gekweekte cellen. Het ging daarbij met name om genen betrokken bij cel-communicatie, cel-adhesie en receptor-activiteit. Voor zes van deze genen, *VCAM1, KIAA1644, FGFR2, COL27A1, IMP-3* en *MME* is het verschil in expressie tussen osteoblasten en fibroblasten nog eens bevestigd met een onafhankelijke methode, real-time RT-PCR. De genen *VCAM1, KIAA1644, FGFR2 en COL27A1* hebben een hogere expressie in osteoblasten, terwijl de genen *IMP-3* en *MME* hoger tot expressie komen in fibroblasten. Omdat de resultaten voor alle zes genen consistent waren voor elk paar (osteoblast *vs*. fibroblast) van de individuele kweken, kan expressie analyse van deze genen gebruikt kan worden als een snelle en efficiënte test om kleine aantallen gekweekte cellen uit botfragmenten te karakteriseren als osteoblasten dan wel als fibroblasten.

Hoofdstuk 3 beschrijft de vergelijking van expressie profielen van gekweekte primaire osteoblasten en osteoblastaire osteosarcoom-cellijnen. Deze vergelijking leverde een significant verschil op in expressie van 286 genen. Functionele annotatie van de eiwitten waarvoor de significant verschillend tot expressie komende genen coderen, liet in osteoblastaire osteosarcoom-cellijnen het volgende zien:

1. een lagere expressie van genen die coderen voor eiwitten die onderdeel zijn van de celmembraan en de extracellulaire matrix;
2. een hogere expressie van genen die coderen voor eiwitten die zich in de celkern bevinden, zoals eiwitten betrokken bij de celdeling en bij herstel van fouten ontstaan tijdens DNA replicatie.

De osteoblastaire osteosarcoom-cellijnen bleken bovendien gemiddeld een lagere expressie te vertonen van het cluster van MMP (matrix-metalloproteinase) genen in het chromosomale gebied 11q22.2 en van het cluster van KAP (keratin-associated protein) genen in 17q21.2. Een gemiddeld verhoogde expressie werd gevonden voor genen van het HOXB cluster gelegen in 17q21.3. Een dergelijke co-expressie van genen zou enerzijds kunnen duiden op een chromosomaal verlies in tumoren en tumor-cellijnen, anderzijds op een gemeenschappelijke regulatie van de transcriptie. Op basis van vergelijking met beschreven chromosomale afwijkingen lijkt in de genoemde gevallen gemeenschappelijke regulatie het meest waarschijnlijk. Om de vraag te kunnen beantwoorden of de gevonden verschillen tussen primaire osteoblasten en osteosarcoom-cellijnen tumor- of cellijn-specifiek zijn, hebben we vervolgens de gen-expressie profielen van primaire osteosarcomen onderzocht.

In hoofdstuk 4 wordt gen-expressie profilering en array-CGH analyse beschreven van drie primaire osteoblastaire osteosarcomen en drie primaire fibroblastaire osteosarcomen. Met behulp van micro-array analyse zijn de expressie-profielen van de primaire osteosarcomen vergeleken met expressie-profielen van osteosarcoom-cellijnen en gekweekte osteoblasten. Individuele vergelijkingen tussen deze drie groepen leverde in totaal 838 significant verschillend tot expressie komende genen op. Functionele annotatie van alle genen laat zien dat de verschillen tussen de primaire osteosarcoom-tumoren enerzijds en gekweekte osteoblasten anderzijds hoofdzakelijk veroorzaakt worden door genen die coderen voor eiwitten die een rol spelen bij ontstekingsprocessen en angiogenese (nieuwe groei van bloed- en lymfevaten). In de osteosarcoom cellijnen komen in vergelijking met de andere twee groepen die genen verhoogd tot expressie die coderen voor eiwitten die te maken hebben met de regulatie van de celdeling. Een onderdrukte expressie van het MMP gencluster werd zowel in primaire osteosarcoom-tumoren als in osteosarcoom-cellijnen geconstateerd. We hebben met behulp van array-CGH ook gekeken naar de chromosomale afwijkingen in zes tumoren. De belangrijkste chromosomale afwijking betreft het verlies van grote delen van de langste arm van chromosoom 13 in 5 van de 6 tumoren. Een verlies van delen van de lange arm van chromosoom 11 (waar het MMP gen cluster op ligt) is alleen in de drie osteoblastaire osteosarcomen geconstateerd, terwijl voor twee van de drie fibroblastaire osteosarcomen er een verlies van gedeelten van chromosomale gebieden op 2p en 8p is gevonden. In totaal zijn er amplificaties gevonden in 13 verschillende chromosomale gebieden (6p21, 6pter, 7p, 8q, 9p, 11q, 12p, 12q13, 12q13-q14, 12q14-q21.3, 16q22.1-q22.2, 17p en 17q). Expressie analyse van genen in deze geamplificeerde gebieden in de tumoren (in vergelijking met de gekweekte osteoblasten) laat zien dat chromosomale amplificatie soms samengaat met verhoogde expressie en dat dit naast genen waarvan de amplificatie reeds bekend was (bv *CDK4*) ook een aantal genen oplevert waarbij amplificatie nog niet eerder is gemeld.

Chapter 7

Dankwoord

Dankwoord

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Marga

Publications

Poelarends GJ, **Wilkens M**, Larkin MJ, van Elsas JD, Janssen DB: Degradation of 1,3-dichloropropene by pseudomonas cichorii 170. Appl Environ Microbiol, *1998*, 64:2931-2936.

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